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METHODS AND MEANS FOR PRODUCING PROTEINS WITH PREDETERMINED POST-TRANSLATIONAL MODIFICATIONS

Abstract:

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(54) **Title:** METHODS AND MEANS FOR PRODUCING PROTEINS WITH PREDETERMINED POST-TRANSLATIONAL MODIFICATIONS

(57) **Abstract:** The present application provides proteins and methods for producing the same, wherein the proteins contain specific post-translation modifications. In particular, the application provides human etythropoietin containing a lower amount of sialic acid residues than epoetin alfa.

Title: Methods and means for producing proteins with predetermined post-translational modifications.

FIELD OF THE INVENTION

The invention relates to the field of medicine. The invention further relates to the production of proteins. More particularly the present invention relates to the production of recombinant proteins for use as a therapeutically active constituent of a pharmaceutical preparation.

BACKGROUND OF THE INVENTION

Recombinant expression systems for the production of proteins are widely known. In general, human recombinant proteins are manufactured with the use of a cellular expression system. These systems range from bacteria, yeast and fungi to plant cells, and from insect cells to mammalian cells. Most of these cell-based systems are only suited for a specific class of proteins. Consideration for the production host and expression system of choice generally relate to ease of use, cost of culturing, growth characteristics, production levels and the ability to grow on serum-free medium. Bacterial systems as well as yeast systems are in many aspects the system of choice, since the cost of culturing is low and they are in general easy to use in comparison to other, higher eukaryotic systems. Nevertheless, it is generally known that prokaryotic- and lower eukaryotic systems differ in a number of aspects from higher eukaryotic systems (such as mammalian cells). For instance, certain post-translational modifications like

glycosylation do no occur in bacterial systems, while many mammalian proteins depend on these post-translational modifications to be therapeutically active. The cellular expression systems mentioned above might also differ in the capacity to exert other co- and post-translation modifications such as folding, phosphorylation, γ-carboxylation, and γ-hydroxylation. In general, the co- and post-translational modifications are cell- and species specific and depend on the expression system being applied.

Until now, Chinese Hamster Ovary (CHO) cells and Baby Hamster Kidney (BHK) cells have been mainly used for the production of recombinant human proteins and peptides for use as a therapeutic active compound. CHO and BHK cells are frequently the expression system of choice when a mammalian expression system is required. These mammalian cells are able to produce proteins efficiently and allow generally for proper processing and folding. More recently, a human cell line was proposed for production of human proteins. The human post-translational modifications of human proteins are thought to have a positive effect on the pharmacokinetic and/or pharmacodynamic properties in general (WO 00/63403).

In the last number of years, studies have revealed more about the complexities of differential post-translational modifications of human proteins and the potential implications on functions in the human body. For example, relatively recent findings suggest that differential glycosylation patterns of human proteins that occur in the blood (so-called 'serum-type' modifications) are different from the ones that occur in the cerebrospinal fluid in the brain ('brain-type' modifications). This difference may be the key issue that is of paramount

importance for the design of effective therapeutics. Taking the difference in post-translational modifications found on proteins that have a function in the serum as well as in the brain as an example, it is now evident that new expression systems are needed that take into account the differential post-translational modifications on therapeutic proteins that are required for a proper activity in different parts of the human body as well as in different types of disease. For example, proteins that might have a beneficial effect on neural disorders should have 'neural-like' post-translational modifications and should be produced on expression systems having the ability to produce proteins harboring such modifications. Proteins that have these specific needs may be beneficial in the treatment of all sorts of disorders, among which are the diseases related to the Central Nervous System (CNS), the peripheral nervous system and heart tissue.

Disorders affecting the CNS encompass different kinds of afflictions such as acute brain damage, neurodegenerative diseases and other dysfunctions such as epilepsy, schizophrenia and mood disorders. With the increase of the number of elderly people in the worldwide population in the next decades, an increasing number of people become at risk of degenerative neurological diseases related to the CNS. Two main examples of such disorders are Parkinson's and Alzheimer's Disease. Other pathological disorders that might afflict neural cells and tissues are due to injuries that might be a result of hypoxia, seizure disorders, neurotoxin poisoning, multiple sclerosis, hypotension, cardiac arrest, radiation or hypoglycemia. Neural injuries might also occur during surgical procedures such as aneurysm repair or tumor resection. To date, the

treatment of the CNS disorders has been mainly via administering pharmaceutical compounds. The main drawback that has been appreciated in the art is the limited ability to transport drugs across the blood-brain barrier and the drug tolerance that is acquired by patients to whom these drugs are administered for prolonged periods of time. Other treatments for degenerative diseases related to the CNS include neurological tissue grafting: the use of fetal cells for neuro-transplantation has been explored. A clear improvement in the condition of Parkinson's patients was reported by several groups after applying such techniques (Freed et al. 1992; Widner et al. 1992). Despite these successful reports, the use of large amounts of aborted fetal tissue for such purposes is hampered by significant problems such as ethical and political issues. Moreover, fetal tissue is most likely never a homogeneous cell population, so it is not a well-defined source of cells, and the question remains whether there will be a sufficient and adequate constant supply of fetal tissue for these purposes. Another method makes use of the fact that in the adult mammalian neural tissue multipotent neural stem cells exist, that are capable of producing progeny that differentiate into neurons and glia (Reynolds and Weiss 1992). Methods have been provided for the proliferation of these stem cells to provide large numbers of neural cells that can differentiate into neurons and glia using various growth factors (U.S. Pat. No. 5,750,376; WO 94/10292). These findings have shown that there might be ways to treat neurological disorders such as degenerative afflictions by the administration of stimulating factors such as recombinant growth factors and/or hormones.

Several investigators have found that nervous tissue expresses high levels of one particular type of hormone, known as Erythropoietin (EPO). Moreover, the EPO-receptor (EPO-R) was also found to be expressed to high concentrations in these tissues (Digicaylioglu et al. 1995; Juul et al. 1997; Marti et al. 1997; Morishita et al. 1997). EPO, a protein famous for its role in differentiating hematopoietic stem cells into red blood cells, also seems to increase the number of neural progeny that are generated from proliferated neural stem cells. Several methods of inducing the differentiation of multipotent neural cells into neurons and methods of treating neurodegenerative diseases or acute brain injuries by producing neurons from such cells using EPO are described in U.S. Pat. No. 6,165,783. Thus, it has recently become clear that EPO not only has a hematopoietic effect, but that there also might be a role for EPO in neural tissues. There is increasing evidence that non-erythroid cells express the EPO-R and respond to recombinant EPO *in vitro* and *in vivo*. For example, neural cell lines like NT2, PC12 and SN6.10.2.2. express the EPO-R, and exposure of PC12 cells to recombinant human EPO causes a rapid influx of calcium from outside the cells and increases the intracellular concentration of monoamines (Masuda et al. 1993). Recombinant human EPO was also found to augment choline acetyltransferase (ChAT) activity in primary cultured mouse septal neurons and in the cholinergic hybridoma cell line, SN6.10.2.2. (Konishi et al. 1993). In the developing and mature brain of rodents, monkeys and humans expression of EPO and the EPO-R has also been detected, (Marti et al. 1996; Dame et al. 2000; Yasuda et al. 1993; Digicaylioglu et al. 1995; Morishita et al.

1997). The expression of EPO and the EPO-R has been localized to neurons and glia cells in spinal cord and brain during fetal human development (Juul et al. 1998). The distribution of EPO and EPO-R proteins in fetal and adult brain has been determined by immunohistochemistry (Juul et al. 1999a and 1999b). The synthesis of EPO in the brain seems to take place primarily in astrocytes (Marti et al. 1986; Masuda et al. 1994) and in neurons (Bernaudin et al. 2000).

Although the emphasis over the last years has been on the role of EPO in erythropoiesis, it was already reported by in 1978 (Peschle et al. 1978) that the production of EPO is not restricted to the fetal liver and adult kidneys, but also takes place inside the developing and adult brain. In the human fetus EPO is expressed at a level that is comparable to that in the liver and kidneys, which are the most relevant sites for circulating EPO at this stage of development (Dame et al. 2001). Also in mice, it has been shown that there is a high constitutive level of EPO mRNA in the brain compared to the kidneys and the liver (Marti et al. 1996). These observations suggested a role of EPO in the development of the CNS (Dame et al. 2001).

EPO protein has also been detected in the cerebrospinal fluid (CSF) of human neonates and adults (Juul et al. 1997; Buemi et al. 2000). The concentration of EPO in CSF is relatively high in neonates and decreases to lower but nevertheless detectable levels in adults (Juul et al. 1997; Marti et al. 1997). Compared to healthy individuals, patients with old cerebrovascular disease and patients with depression have an elevated level of EPO in the CSF (Nakamura et al. 1998). That EPO is produced locally in the brain is strengthened by the observation

that EPO does normally not cross the intact blood-brain barrier because there is no correlation between the concentration of EPO in the serum and in the CSF (Marti et al. 1997). This was further confirmed by the fact that the intravenous administration of a total dose of 6,000 U of recombinant human EPO in a human patient did not lead to an increased concentration of EPO in the CSF (Buemi et al. 2000). Yet, studies in mice and rats have shown that EPO is able to cross the blood-brain barrier when recombinant human EPO is applied systemically (intra-peritoneal) at a relatively very high dose of 5,000 U/kg body weight (Brines et al. 2000; WO 00/61164). EPO also seems to be transported into the brain in case of blood-brain barrier dysfunction, e.g., in case of a traumatic brain injury. In that case, a correlation between the levels of EPO in serum and CSF is found (Marti et al. 1997).

Interestingly, the expression of the EPO gene in the kidneys as well as in the brain is increased under hypoxic conditions. The expression is regulated by the transcription factor hypoxia-inducible factor-1, which is activated by a variety of stress signals, including hypoxia. It has recently been demonstrated in mice that the EPO mRNA levels markedly increase within 4 hours upon the exposure to hypoxia (Chikuma et al. 2000). Yet, the EPO mRNA levels in the kidneys decreased within 8 hours during continuous hypoxia whereas the EPO mRNA levels in the cerebrum remained the same. Hence, the regulation of EPO expression is tissue-specific, which further strengthens the hypothesis that EPO has tissue-specific functions that are different between the brain and the bone marrow (Masuda et al. 1999; Chikuma et al. 2000; Sasaki et al. 2001), and that therefore EPO, besides its heamatopoietic function,

also has other functions such as a neurotrophic role. The expression of EPO in monkey- and mouse brain has been shown to be 3 to 20-fold increased respectively, under hypoxic conditions (Digicaylioglu et al. 1995; Marti et al. 1996). Hypoxic effects on the expression of EPO have also been demonstrated *in vitro*. In primary astrocyte cultures hypoxia caused a more than 100-fold up-regulation of the mRNA levels of EPO (Marti et al. 1996). Furthermore, it has been found that the mRNA level of the EPO-R is also upregulated under hypoxic conditions *in vivo*. This was found in studies in which the middle cerebral artery of rats was occluded and in which the increase of EPO-R mRNA was detected by *in situ* hybridization in the periphery of a cerebrocortical infarct (Sadamoto et al. 1998). It suggests that neurons increase their sensitivity to EPO by increasing their number of EPO-R under hypoxic and/or ischemic conditions. Finally, it has recently been confirmed by immunohistochemical methods that the expression of immuno-reactive EPO and EPO-R is also upregulated in fresh infarcts inside the human ischemic/ hypoxic brain (Sirén et al. 2001).

Several studies have now demonstrated that EPO can act as a neurotrophic factor. Neurotrophic factors are defined as humoral molecules acting on neurons to influence their development, differentiation, maintenance, and regeneration (Konishi et al. 1993). Neurotrophic effects of EPO have first been shown *in vitro*, in cultured neurons. For example, it has demonstrated that recombinant human EPO, in a dose-dependent manner, protects cultured embryonic rat hippocampal and cerebral cortical neurons from glutamate toxity (Morishita et al. 1997). Hypoxia-induced cell death in cultures of postnatal rat hippocampal neurons also has

been shown to be reduced by EPO (Lewczuk et al. 2000). In addition, it has been shown that recombinant mouse EPO protects cultured rat cortical neurons, but not astroglia from glucose deprivation-induced hypoxia and from the neurotoxic effects of (±)-α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (Sinor and Greenberg 2000). Furthermore, recombinant human EPO has recently been proven not only to protect neural cell cultures form hypoxia but also from serum deprivation or kainic acid exposure (Sirén et al. 2001). The neurothropic function of EPO has also been shown in rats, which were treated with soluble EPO-R that competed with the natural EPO-R for binding endogenous EPO. These rats showed neural degeneration and an impaired learning ability (Sakanaka et al. 1998). The neurotrophic and neuroprotective effects of EPO have also been studied in animal models with hypoxic-ischemic injuries. For example, it has been shown that the infusion of recombinant human EPO into the lateral ventricles of gerbils prevented ischemia-induced learning disability and rescued hippocampal CA1 neurons from lethal ischemic damage in a dose-dependent manner (Sakanaka et al. 1998). In addition, it has been found that recombinant human EPO, which was infused into the cerebroventricles of stroke-prone spontaneously hypertensive rats with permanent occlusion of the left middle cerebral artery, alleviated the ischemia-induced place navigation disability and supported neuron survival (Sadamoto et al. 1998). In a similar model, it has been shown that systemic administration (intra-peritoneal) of a high dose (5,000 U/kg body weight) of recombinant human EPO (Epoietin alpha, Procrit, Ortho Biotech, Raritan, NJ) reduced the infarct volume by ~75% at 24 h after occlusion of the middle-

cerebral artery (Brines et al. 2000). In the same study, it was also observed that the treatment with EPO reduced immune damage in experimental autoimmune encephalomyelitis, as well as kainate-induced toxicity. Furthermore, the effects of EPO have been studied in rabbits with subarachnoid hemorrhage-induced acute cerebral ischemia (Alafaci et al. 2000). It was found that recombinant human EPO that was systemically administered after the subarachnoid hemorrhage significantly reduced the number of necrotic cortical neurons compared to the placebo controls.

In addition to the mentioned effects of EPO on erythropoiesis and neuroprotection, other roles of EPO have been described. The expression of EPO and its receptor are identified in endothelial cell *in vitro* and *in vivo* (Anagnostou et al. 1994). *In vitro* experiments demonstrated that recombinant human EPO stimulates cell migration and proliferation in endothelial cell cultures. These *in vitro* results were further extended by the effect of human recombinant EPO on the stimulation of new blood vessel formation in the chick chorioallantoic membrane (Ribatti et al. 1999). In accordance in mice, EPO was found to play an important role in the 17-beta-estradiol dependent angiogenesis in the uterine endometrium (Yasuda et al. 1998). Proliferative effects of EPO have also been shown on muscle cells. In the myoblast C2C12 cell-line EPO enhanced the proliferation and reduced the differentiation and fusion into myotubes of these cells *in vitro* (Ogilvie et al. 2000). Furthermore, the receptor of EPO could also be detected on primary satellite cells isolated from skeletal muscle from mice and on C2C12 cells at the mRNA and at the protein level. In addition EPO has been shown to stimulate

DNA-synthesis and proto-oncogene expression in rat vascular smooth muscle cells.

An increased proliferation following recombinant human EPO administration was also found on primary cultures of neonatal rat cardiac myocytes (Wald et al. 1996). This mitogenic effect of EPO was associated with a stimulatory effect on Na^+-K^+-ATPase activity and was shown to be secondary to the activation of tyrosine kinase and PKC. These enzymatic pathways have also been described for other cytokines in different tissues. A role for EPO on the proliferation of cardiomyocytes was supported by knockout studies. During embryogenesis EPO is expressed in the heart and mice lacking EPO or EPO-R expression display cardiac defects, demonstrated by ventricular hypoplasia and a reduction in the number of proliferating cardiomyocytes and increased apoptosis (Wu et al. 1999). Yu et al. (2001) demonstrated that the cardiac phenotype in the EPO-R knockouts could be rescued by crossing these mice with transgenic mice, harboring the human EPO receptor gene. In addition to the restoration of erythropoiesis, the cardiac defect was corrected and apoptosis was markedly reduced. Furthermore, also apoptosis in liver and brain was significantly reduced in these mice.

Recent clinical studies demonstrated the beneficial effects of EPO in patients with congestive heart failure (CHF). CHF is defined as a heart disease, in which the heart is not able to pump blood at a rate required by the metabolizing tissues, or when the heart can do so only with an elevated filling pressure. A high percentage of CHF patients are anemic (low hemaglobin percentage) and a correlation exists between the severity of the condition of CHF and the degree of anemia. When patients with anemia in

CHF were treated with EPO, a significant improvement with respect to cardiac function, renal function and a marked decrease was observed in the need for diuretics and hospitalization (Silverberg et al. 2000 and 2001). Although the beneficial effects of EPO have been ascribed to its effect on erythropoiesis, direct cardiac effects, such as anti-apoptosis and angiogenesis may partially explain the positive outcome in these patients.

During ischemia/reperfusion injury, decreased cellular oxidative phosphorylation leads to the failure of energy-rich phosphates, followed by an altered membrane potential, production of reactive oxygen species, cytokines and repression of protective gene products such as Nitric Oxide Synthase. In this process several adhesion molecules are expressed on the endothelial cells in the affected area. These promote leukocyte adhesion to the endothelium followed by transmigration. Transmigration and subsequently activation of leukocytes leads to the production of toxic reactive oxygen species, proteases, elastases and is generally followed by apoptosis, or other types of cell death (Carden and Granger 2000; Butcher 1991; Panes et al. 1999).

A certain family of glycoproteins, named selectins, play an important role in the initial steps of adhesion of leukocytes to the endothelium in ischemia/reperfusion injury. There are three members in the selectin family: P-selectin, E-selectin and L-selectin. L-selectin is constitutively expressed on leukocytes, whereas P-selectin and E-selectin are found on the membrane upon activation. P-selectin is present in Weibel-Palade bodies of endothelial cells and alpha-granules of platelets. Following ischemia/reperfusion or activation by different

agents, P-selectin is translocated to the cell surface, within 10-20 minutes (Lorant et al. 1991; Weyrich et al. 1995), whereas E-selectin is expressed on endothelial cells after *de novo* synthesis, which takes approximately 4-6 hours (Bevilacqua et al. 1989). Selectins initiate the rolling of the leukocytes along the endothelium. P-selectin is the most important selectin for this first step of leukocyte rolling (Lefer and Lefer 1996). The rolling of the leukocytes reduces the velocity of the leukocytes in the bloodstream and allows a more firm interaction between the leukocytes and the endothelium by other adhesion molecules (integrins). Firm adhesion is followed by transendothelial migration. Infiltration of the neutrophils can be observed 3 hours following reperfusion. After this time period, reperfusion injury with its resulting cell death takes place (Armstead et al. 1997). Several ligands of selectins have been identified. P-selectin glycoprotein ligand-1 (PSGL-1) is a high affinty ligand for P-selectin and to a lesser extent for L-selectin and E-selectin (Moore et al. 1994). The oligosaccharides in PSGL-1 are recognized by the lectin domain of the selectins.

It has been well established in the art that the function of (recombinant) EPO depends heavily on the glycosylation pattern of the oligosaccharides present on the protein. The N-linked oligosaccharides of human EPO are highly important for its well-known biological activity: the stimulation of erythropoiesis (Takeuchi and Kobata 1991; Wasley et al. 1991). For instance, the sialic acids protect the protein from being cleared through the hepatic asialoglycoprotein receptor (Tsuda et al. 1990; Morimoto et al. 1996). Furthermore, the highly branched N-linked oligosaccharides are thought to increase the volume of the

protein such that it is not easily filtered by the kidney (Takeuchi et al. 1989; Misaizu et al. 1995). The multi-antennary sugar structures may also have a role in targeting of EPO to the bone marrow where erythropoiesis occurs (Takeuchi et al. 1989). The role of the O-linked oligosaccharides present on EPO is relatively unclear and some data have suggested that there is only a limited role for the O-linked sugar in EPO present in circulation and that has an effect on hematopoiesis.

In general, human neural glycoproteins are characterized by their glycosylation, which has been referred to in literature as 'brain-type' glycosylation (Margolis and Margolis 1989; Hoffmann et al. 1994). In contrast to 'serum-type' glycosylated proteins (i.e., glycoproteins circulating in the blood) brain-type glycosylated proteins characteristically possess complex-type N-linked sugars that are modified with α1,3-linked fucose attached to N-acetyl-glucosamine in lactosamine-type antennae thereby forming Lewis x or sialyl-Lewis x structures (Fig. 5). There are two types of Lewis x structures: One with a terminal galactose residue and one with a terminal N-acetyl-galactosamine (GalNac) residue. If these terminal groups are linked to a sialic acid, the Lewis x structure is called a sialyl Lewis x structure. Another difference between serum-type and brain-type oligosaccharides is that the latter often contain terminal N-acetyl-glucosamine and/or terminal galactose, and may include a terminal N-acetyl-galactosamine modification, whereas serum-type oligosaccharides usually contain only low amounts of such structures. It has also been suggested that brain-type N-oligosaccharides characteristically

contain high amounts of bisecting N-acetyl-glucosamine (Hoffmann et al. 1994).

It is thus far unknown what the O-glycan structure is in brain-type EPO, but the limited role for this oligosaccharide in serum might imply that there is a more important role for this type of glycosylation in brain-type EPO. It is very likely as well, that in accordance with the N-glycans, also the O-glycan has a differential glycosylation pattern between serum-type and brain-type.

Oligosaccharides that are generally found on proteins circulating in the serum contain often heavily galactosylated structures. This means that a galactose is linked to a peripheral N-acetyl-glucosamine thereby forming a lactosamine structure. The glycoprotein is in this way protected from endocytosis by the N-acetyl-glucosamine receptors (i.e., receptors that recognize terminal N-acetyl-glucosamine) present in hepatic reticuloendothelial cells and macrophages (Anchord et al. 1978; Stahl et al. 1978). Serum-type oligosaccharides usually also contain terminal sialic acids (also often referred to as neuraminic acid) which protect the glycoprotein from clearance through the asialoglycoprotein receptor. These clearance mechanisms specifically apply to glycoproteins circulating in the blood and are probably lacking in the human central nervous system (CNS) (Hoffmann et al. 1994). A difference in glycosylation is exemplified by transferrin that occurs in significant amounts as asialotransferrin in the CSF but not in that form in serum (Van Eijk et al. 1983; Hoffmann et al. 1995). Transferrin is a protein capable of interacting with iron via two iron-binding sites in the protein. Iron uptake by cells occurs through receptor-mediated endocytosis of the transferrin protein that is bound to

iron. Human serum-type transferrin carries two N-glycosylation sites (Asn413 and Asn611) that are generally occupied by disialylated, bi-antennary oligosaccharide chains. Most transferrin proteins seem to have 4 sialic acid residues (i.e. two on each of the bi-antennary chains). Serum-type transferrin does not contain polylactosamines or fucose residues. A minor amount of serum-type transferrin carries sialylated tri-antennary oligosaccharides. It has been found that rarely also tetra-antennary structures occur.

The glycosylation of transferrin is used as a marker for carbohydrate-deficient glycoprotein syndromes (CDGS). As a result there is extensive information in the literature regarding glycosylation status. This includes information of transferrin isolated from various human sources including serum, cerebrospinal fluid (CSF), amniotic fluid and synovial fluid. Apparently differentially glycosylated forms are present in separate parts of the human body strongly suggesting a differential role of the different glycosylation patterns present on transferrin in these separate tissues, as has been discussed here for EPO.

In the case of EPO, one can also refer to a serum-type EPO (or a 'renal-type', or a 'urinary-type' EPO) for the protein that is produced in the kidney and that circulates in the blood, as compared to EPO that is been produced by other tissues such as the brain (brain-type).

EPO protein that was produced by cultured rat brain cells was found to be significantly smaller than the EPO protein present in circulation. This mass difference might be the reason for the different biological roles in the brain and in circulation. It was found, that although

brain-type EPO produced on these cultured rat brains is approximately 15% smaller than serum-type EPO (presumably mainly due to differences in sialylation), this brain-type EPO is more active *in vitro* in erythroid colony stimulation at low ligand concentrations (Masuda et al. 1994). The reason for this finding might be that, although the affinity for brain-type EPO is higher towards the EPO-R, the brain-type EPO will in its specific glycosylation form never circulate long enough *in vivo* to exert the effects that are normally brought about by the serum-type EPO because this type of EPO is better protected against kidney filtering and binding to the hepatic asialoglycoprotein receptors, as discussed above. It is also generally known by persons skilled in the art, that a low sialylation of the EPO protein is beneficial for its affinity towards the EPO-R. However, for circulating EPO involved in erythropoiesis it is better to be protected against clearance by heavy sialylation, than have a very high affinity for the receptor.

There is a possible role for the sialyl Lewis x modifications in oligosaccharides in binding to selectins (Foxall et al. 1992). Sialyl Lewis x structures are expressed on leukocytes and are rapidly expressed on vascular endothelial cells and cardiac myocytes following myocardial ischemia/reperfusion injury *in vivo* (Yamazaki et al. 1993). Furthermore, sialyl Lewis x structures are also induced on the surface of endothelial cells and cardiomyocytes by hypoxia/reoxygenation *in vitro* (Seko et al. 1996). Several studies in the art have indicated the importance of selectins and sialyl Lewis x structures for the adhesion of leukocytes in models of ischemia/reperfusion. The sialyl Lewis x oligosaccharide Sle^x-OS was

shown to be cardioprotective in a feline model of ischemia/reperfusion by reducing cardiac necrosis by 83% (Buerke et al. 1993). In addition reduced adhesion to the endothelium was observed in this model using Sle^x-OS. Furthermore, in a similar ischemia/reperfusion model, treatment with Sle^x-OS also resulted in a 100% recovery in cardiac function, compared to 71% recovery of cardiac function with saline. In dogs subjected to myocardial infarction/reperfusion, treatment with Sle^x-OS demonstrated cardioprotection by a 55% reduction in infarct size (Flynn et al. 1996).

For many years a recombinant version of the serum-type EPO has been used in patients suffering from kidney failure, anemia and/or in patients undergoing heavy surgery resulting in dramatic blood-loss. Generally, this recombinant EPO results in an increased production of red blood cells from hematopoietic stem cells in the bone marrow. It is well established in the art that the recombinant EPO had to fulfill all requirements of a stable protein that could circulate in the bloodstream for a sufficient amount of time to enable the induction of erythropoiesis. The system of choice thus far has been a production platform on CHO cells from which the higher sialylated EPO forms were purified and used to prepare a medicament for the treatment of patients that suffer from the disorders resulting from a low-red blood cell level. Other cells that were used for these purposes were BHK cells. Apparently CHO and BHK cells are capable of generating the correct glycosylation (sialylation) patterns on the recombinant product to yield positive effects in human renal-failure patients. However, CHO produced EPO does not have the characteristic features of an EPO

molecule that is active in the brain or in tissues that involve selectin-based transport. Therefore, urinary- or serum-type EPO (produced on cells such as CHO or BHK) is relatively useless in the treatment of disorders related to the Central- or Peripheral Nervous system as well as in the treatment of afflictions related to ischemia/reperfusion induced disorders. This, because of its glycosylation pattern that is not suited for these kind of tissues, and also because it renders side effects such as an increase in the number of red blood cells (erythropoiesis) due to its strong hematopoietic activity.

No proper production platforms are present in the art that are able to produce significant amounts of recombinant proteins harboring tissue-specific predetermined post-translational modifications such as a brain-type glycosylation on recombinant EPO, and that can be used in the manufacturing of medicaments for the treatment of patients suffering from disorders that require such proteins, as well as the treatment of patients at risk of developing such disorders.

BRIEF DESCRIPTION OF TABLES AND FIGURES

Table I. Overview of the marker proteins that can be used to characterize cells.

Table II. Positive control tissues that can be used for some of the marker proteins depicted in Table I.

Table III. Detailed information (Supplier and Catalogue numbers) of antibodies directed to marker proteins that were used to characterize the PER.C6™ cell line.

Table IV. Score of the presence of the marker proteins on PER.C6.

Table V. Monosaccharide composition of the N-linked sugars of PER.C6-EPO and Eprex.

Table VI. Assignments of MS peaks observed for the molecular ions of desialylated N-glycans released by N-glycanase F from EPO produced in DMEM by EPO producing PER.C6 clone P7. Peaks with mass (m/z) values that are also found in Eprex are underlined and indicated in bold.

Table VII. Assignments of MS peaks observed for the molecular ions of desialylated N-glycans released by N-glycanase F from EPO produced in DMEM by EPO producing PER.C6 clone P8. Peaks with mass (m/z) values that are also found in Eprex are underlined and indicated in bold.

Table VIII. FUT activities in CHO and PER.C6 cells.

Figure 1. Mass spectra of the N-linked sugars of Eprex, P7-EPO (pools A, B,and C), and P8-EPO (pools A, B, and C). (A) Eprex; (B) P7, pool A; (C) P7, pool B; (D) P7, pool C; (E) P8, pool A; (F) P8, pool B; and (G) P8, pool C.

Figure 2. Sialic acid content of PER.C6-EPO and CHO-EPO.

Figure 3. Lewis x glycan structures present on PER.C6-EPO.

Figure 4. Lewis x structures expression at the PER.C6 cell surface.

Figure 5. Schematic representation of Lewis x and Sialyl Lewis x structures.

Figure 6. Effect of PER.C6-EPO and Eprex on erythropoiesis *in vivo*.

Figure 7. Infarct volumes in untreated rats (control) and Eprex and PER.C6-EPO treated rats based on the ADC maps (Fig. 7A) and the T2 maps (Fig. 7B) generated at 24 h after the onset of reperfusion, using MRI.

Figure 8. Concentration of Eprex at the indicated time-points after a single i.v. injection of 150 eU of Eprex in three animals.

SUMMARY OF THE INVENTION

The present invention provides methods for identifying, selecting and obtaining mammalian cells that are capable of producing proteinaceous molecules, such as peptides and proteins comprising post-translational modifications, wherein said post-translational modifications are predetermined and brought about by the mammalian cell in which the proteinaceous molecule is expressed. The invention further provides methods for obtaining and producing proteinaceous molecules, such as erythropoietin (EPO), using mammalian cells obtainable according to methods of the present invention and on mammalian cells that have been obtained on the basis of their ability to produce proteins and/or post-translational modifications that are elusive for the predetermined post-translational modification that is desired.

In one preferred embodiment, the present invention provides mammalian cells that have neural characteristics and properties such that significant amounts of recombinant proteins can be produced that harbor 'neural- or brain-type' properties. The production of recombinant proteins, like brain-type EPO, carrying specific predetermined post-translational modifications, is now feasible by using the methods and means of the present invention.

The present invention furthermore provides methods for purifying proteinaceous molecules, wherein said proteinaceous molecules are purified from cell culture on the basis of the predetermined post-translational modification present on the molecule, said predetermined post-translational modification being brought about by the mammalian cell on which the molecule was produced.

The present invention furthermore provides for use of a composition of erythropoietin-like molecules selected from the group consisting of one or more muteins of erythropoietin, one or more derivatives of erythropoietin, or a collection of one or more fractions of erythropoietin molecules sialylated to a varying degree, for the preparation of a medicament for the treatment of a disorder selected from the group consisting of ischemia, a reperfusion injury, a hypoxia-induced disorder, an inflammatory disease, a neurodegenerative disorder, and acute damage to the central- or peripheral nervous system, wherein said composition of erythropoietin-like molecules has on a protein content basis a lower erythropoietic activity *in vivo* than epoetin alfa. The present invention also provides pharmeceutical compositions comprising such erythropoietin-like molecules. The invention also provides methods for treatment or prevention said disorders, comprising administering said compositions.

DETAILED DESCRIPTION

The present invention provides a method for identifying a mammalian cell capable of producing a proteinaceous molecule comprising a predetermined post-translational modification, said method comprising the steps of: a) analyzing the post-translational modification on a protein produced by said mammalian cell; and b) determining whether said protein comprises said predetermined post-translational modification.

In another embodiment the invention provides a method for selecting a mammalian cell capable of producing a

proteinaceous molecule comprising a predetermined post-translational modification, said method comprising the steps of: a) analyzing the presence or absence of a tissue specific marker or a combination of tissue specific markers in said mammalian cell or on the cell surface of said mammalian cell, which marker or combination of said markers is indicative for said predetermined post-translational modification to be present on said proteinaceous molecule; and b) selecting said mammalian cell on the basis of the presence or absence of said tissue specific markers.

In yet another embodiment, the invention provides a method for obtaining a mammalian cell from a heterogeneous cell population, said mammalian cell being capable of producing a proteinaceous molecule comprising a predetermined post-translational modification, said method comprising the steps of: a) sorting cells on the basis of the post-translational modifications on proteins produced by said cells in said heterogeneous cell population; and b) selecting the cells capable of producing proteins comprising said predetermined post-translational modification.

In another embodiment, the invention provides a method for identifying a mammalian cell capable of producing a proteinaceous molecule comprising a predetermined post-translational modification, said method comprising the steps of: providing said mammalian cell with a nucleic acid encoding a protein capable of comprising post-translational modifications, in such a way that said mammalian cell harbors said nucleic acid in an expressible form; culturing said mammalian cell under conditions conducive to the production of said protein; analyzing the post-translational modification on said protein produced by said

mammalian cell; and determining whether said post-translational modification present on said protein comprises said predetermined post-translational modification. Whereas in yet another embodiment, the invention provides a method for identifying a mammalian cell capable of producing a proteinaceous molecule comprising a predetermined post-translational modification, said method comprising the steps of: providing said mammalian cell with a nucleic acid encoding said proteinaceous molecule capable of comprising post-translational modifications, in such a way that said mammalian cell harbors said nucleic acid in an expressible form; culturing said mammalian cell under conditions conducive to the production of said proteinaceous molecule; analyzing the post-translational modification on said proteinaceous molecule produced by said mammalian cell; and determining whether said post-translational modification present on said proteinaceous molecule comprises said predetermined post-translational modification.

A proteinaceous molecule as used herein refers to, but is not limited to, molecules such as peptides, polypeptides and proteins, as well as to mutants of peptides, polypeptides and proteins (molecules comprising deletions, pointmutations, swaps and/or chemically induced alterations). It also refers to peptides, polypeptides and proteins carrying tags and/or other proteinaceous and non-proteinaceous labels (e.g., radio-active compounds). An example of such a protein is human EPO, which has besides the renal- or serum-type form, other phenotypes such as a brain-type form. Other, non-limiting examples of classes of proteins that have certain characteristics that possibly play an important role in the functionality of the protein

in certain tissues and that should (when recombinantly expressed) harbor the predetermined post-translational modifications for a proper function are monoclonal antibodies, neurotrophins, cytokines, insulin-like growth factors, TGF-β like growth factors, fibroblast growth factors, epidermal growth factors, heparin binding growth factors, tyrsosine kinase receptor ligands and other trophic factors. Most of these factors are associated with disease syndromes, and therefore most of the proteins might be used in recombinant form in the treatment of humans, provided that the proteins harbor the post-translational modifications necessary to be active *in vivo*. These proteins should therefore be produced on expression systems that are capable of providing the required post-translational modifications. Examples of such proteins are, but are not limited to, transferrin, Nerve Growth Factor (NGF), Brain-derived neurotrophic factor, Neurotrophin-3, -4/5 and -6, Ciliary neurotrophic factor, Leukemia inhibitory factor, Cardiotrophin-1, Oncostatin-M, several Interleukins, GM-CSF, G-CSF, IGF-1 and -2, TGF-β, Glial-derived neurotrophic factor, Neurturin, Persephin, Myostatin, Fibroblast Growth Factor-1, -2 and -5, Amphiregulin, Acetylcholine receptor inducing activity, Netrin-1 and -2, Neuregulin-2 and -3, Pleiotrophin, Midkine, Stem Cell Factor (SCF), Agrin, CSF-1, PDGF and Saposin C. Monoclonal antibodies refer to human and humanized antibodies, to parts thereof, and to equivalents such as single chain Fv (scFv) fragments, Fab fragments, CDR regions, variable regions, light chains and heavy chains. Antibodies may be bispecific, trispecific, and so forth; either in naked form or conjugated to antigenic

moieties, toxins, fluorescent markers, radiolabels, and the like.

A post-translational modification as used herein refers to any modification that is present on or in said proteinaceous molecule. It refers to modifications that are introduced during or subsequent to the translation of said molecule from RNA *in vivo* or *in vitro*. Such modifications include, but are not limited to, glycosylation, folding, phosphorylation, γ-carboxylation, γ-hydroxylation, multimerization, sulphide bridging and for instance processing events such as the clipping-off or the addition of one or more amino acids. A predetermined post-translational modification as used herein refers to any post-translational modification that is useful for the selected treatment. According to a preferred embodiment, predetermined post-translational modification refers to a form of modification that makes the modified protein particularly useful to treat disorders of specific tissues, organs, compartments and/or cells of a human or animal body. Preferably, the proteinaceous molecule carrying such predetermined post-translational modification is less active in a tissue, organ, compartment and/or cell wherein action is less desired. Even more preferred, the proteinaceous molecule carrying such predetermined post-translational modifications does not exert any significant effect (such as detrimental- or other undesired side-effects) other than the tissue, organ, compartment and/or cell that is to be treated. According to one embodiment, the predetermined post-translational modification causes the protein comprising the predetermined post-translational modification to be cleared from the blood more rapidly, e.g., to reduce adverse side effects. The predetermined

post-translational modification can be fully understood in detail in advance, but can also be generally referred to as being a desired state that is required for a proper and wanted activity of the proteinaceous molecule comprising such predetermined post-translational modification, meaning that the detailed modifications present on the proteinaceous molecule of interest might not be fully understood and/or defined, but nevertheless hold activity features that are desired. The several glycosylation modifications in O- and/or N-glycans that might be present on a proteinaceous molecule, that might, but do not have to be necessarily desired, are exemplified by structures such as Lewis x, sialyl Lewis x, GalNac, GlcNac, LacdiNAc, α1,3-linked fucose attached to N-acetyl-glucosamine, terminal N-acetyl-glucosamine, terminal galactose, bisecting N-acetyl-glucosamine, sulphate group and sialic acid.

The mammalian cells of the present invention are preferably human, for the production of human proteins to produce proteins that most likely carry mammalian-, and preferably human, characteristics. To produce proteinaceous molecules that should have neural post-translational modifications, it is preferred to use cells that have neural characteristics, such as protein markers that are indicative for neural cells. This does not exclude that a non-neural cell might be extremely useful in producing proteins comprising neural-type post-translational modifications. It depends on the protein activity that is required, to select, identify or obtain a cell that is capable of producing such post-translational modifications. The presence of certain cell-type specific marker proteins in or on a cell does also not exclude the possibility that a certain type of cell is capable of producing a protein

carrying the desired post-translational modifications, although this cell expresses some unrelated protein markers. It depends on the selection criteria, as well as on the method for selecting (determining the protein markers and/or the post-translational modifications present on proteins produced in the cell) whether a certain cell is suitable for producing the proteinaceous molecule of interest.

Since it is required to produce large quantities of proteins when these will be applied in therapeutic settings, it is preferred that the mammalian cells of the invention are immortalized. Immortalization can be brought about in many ways. Examples of methods to obtain immortalized cells are actively transforming a resting cell into a dividing cell by the addition of nucleic acids encoding transforming and/or immortalizing proteins, or through chemical treatment through which endogenous proteins might become transforming, or by taking cells from tumor material. One preferred method to immortalize non-tumorous cells is by the addition of the E1 region of adenovirus as was shown for cell lines such as HEK293, 911 and PER.C6. Also the presence of certain Human Papillomavirus (HPV) proteins might render a cell immortalized (HeLa cells). The addition of certain viral proteins, such as E1 from adenovirus might be beneficial for the production of recombinant proteins, since many of such proteins have transcription-activating features, as well as anti-apoptotic effects. Moreover, they might add to the post-translational modifications in and/or on the proteinaceous molecules that are produced in the cells in which these transforming/immortalizing proteins are expressed. For instance, E1A of adenovirus might induce the

expression of certain endogenous proteins that have a role in the addition of post-translational modifications on the recombinant proteins to be produced. Moreover, a direct effect in these processes can also not be excluded.

The present invention provides a method for producing a proteinaceous molecule comprising a predetermined post-translational modification, comprising the steps of: providing a mammalian cell obtainable by methods according to the invention, with a nucleic acid encoding said proteinaceous molecule in such a way that said mammalian cell harbors said nucleic acid in an expressible form; and culturing said mammalian cell under conditions conducive to the production of said proteinaceous molecule.

In one embodiment of the invention, the invention provides a method for producing a proteinaceous molecule comprising a predetermined post-translational modification, comprising the steps of: identifying a mammalian cell having the ability to provide the proteinaceous molecule with said predetermined post-translational modification; providing said mammalian cell with a nucleic acid encoding said proteinaceous molecule in such a way that said mammalian cell harbors said nucleic acid in an expressible form; and culturing said mammalian cell under conditions conducive to the production of said proteinaceous molecule.

In another embodiment, the invention provides a method for producing a proteinaceous molecule comprising a predetermined post-translational modification, said method comprising the steps of: identifying a mammalian cell having the ability to provide said proteinaceous molecule with said predetermined post-translational modification; providing said mammalian cell with a nucleic acid encoding said proteinaceous molecule in such a way that said

mammalian cell harbors said nucleic acid in an expressible form; culturing said mammalian cell under conditions conducive to the production of said proteinaceous molecule; analyzing said post-translational modifications on said proteinaceous molecule so produced; and determining whether said post-translational modification present on said proteinaceous molecule comprises said predetermined post-translational modification. A suitable cell line for the methods for producing proteinaceous molecules according to the invention is PER.C6, deposited under No. 96022940 at the European Collection of Animal Cell Cultures at the Center for Applied Microbiology and Research.

The invention moreover provides methods for producing a proteinaceous molecule comprising a predetermined post-translational modification, said method comprising the steps of: providing a mammalian cell obtainable by a method according to the present invention, with a nucleic acid encoding said proteinaceous molecule in such a way that said mammalian cell harbors said nucleic acid in an expressible form; culturing said mammalian cell under conditions conducive to the production of said proteinaceous molecule, and purifying said proteinaceous molecule from the mammalian cell culture.

In another embodiment, the present invention provides methods for producing a proteinaceous molecule comprising a predetermined post-translational modification, said method comprising the steps of: providing a mammalian cell obtainable by a method according to the present invention, with a nucleic acid encoding said proteinaceous molecule in such a way that said mammalian cell harbors said nucleic acid in an expressible form; culturing said mammalian cell under conditions conducive to the production of said

proteinaceous molecule; analyzing said post-translational modifications on said proteinaceous molecule so produced; and determining whether said post-translational modification present on said proteinaceous molecule comprises said predetermined post-translational modification.

Preferably, said methods for producing proteinaceous molecules comprise the extra step of purifying said proteinaceous molecule from the mammalian cell culture. More preferred are methods for producing a proteinaceous molecule in a mammalian of the invention, wherein said mammalian cell is immortalized, and wherein said immortalization is brought about as discussed above. Immortalization can take place prior to the identification of the obtained mammalian cell, but might also take place after the proper cell for proper post-translational modifications is identified, selected and/or obtained.

Purification as used herein might be performed by using conventional methods that have been described in the art, however, it is preferred to use purification methods that comprise a step in which the post-translational modifications present in and/or on said proteinaceous molecules are employed. Even more preferred are purification methods that comprise a step in which the predetermined post-translational modifications present in and/or on said proteinaceous molecules are employed. When affinity purification methods are applied, it is preferred to use antibodies or other binders, such as lectins specific for particular carbohydrate moieties and that are directed against certain types of post-translational modifications. Examples of such antibodies are antibodies directed against (sialyl) Lewis x structures, lacdiNac

structures or GalNac Lewis x structures. Using such antibodies enables one to purify the (recombinant) proteins such that a high percentage of the purified protein carries the desired predetermined post-translational modification. Even more preferred are methods in which the proteinaceous molecule is purified to homogeneity. Examples of methods for purification of proteins from mammalian cell culture are provided by the present invention and encompass for instance affinity chromatography methods for the purification of brain-type glycosylated EPO by using antibodies directed against Lewis x structures present in the N-glycans of the recombinantly produced product.

The present invention provides a pharmaceutically acceptable composition comprising a proteinaceous molecule having a predetermined post-translational modification, obtainable according to methods of the present invention, and a pharmaceutically acceptable carrier. Pharmaceutically acceptable carriers as used herein are exemplified, but not limited to, adjuvants, solid carriers, water, buffers, or other carriers used in the art to hold therapeutic components, or combinations thereof. In a preferred embodiment said proteinaceous molecule in said pharmaceutically acceptable composition is erythropoietin. Even more preferred, said erythropoietin has a lower erythropoietic effect as compared to erythropoietin not having said predetermined post-translational modification. According to the invention, erythropoietin produced in cells with neural protein markers acquires a post-translational modification that is active in neural tissue or on neural cells. However, the post-translational modifications are not comparable to the post-translational modifications seen on EPO that circulates in the blood. The

erythropoietic effects of the EPO produced on cells with the neural protein markers is significantly lower, most likely due to the absence of a high percentage of sialic acids, and/or to the presence of brain-type features such as Lewis x structures and terminal galactosides. This is advantageous, since such a brain-type EPO can be used in relatively high dosages in the treatment of disorders related to neural tissue or in the treatment of tissue damaged by ischemia (such as an ischemic heart), but that does not has any significant effect on erythropoiesis. Such disadvantageous effects are appreciated in the art (Wiessner et al. 2001). The invention provides recombinant erythropoietin comprising at least one post-translational modification selected from the group consisting of: a sialyl Lewis x structure, a Lewis x structure, a α1,3-linked fucose attached to N-acetyl-glucosamine, a LacdiNAc structure, a terminal N-acetyl-glucosamine group and a terminal galactose group. Preferably, said recombinant erythropoietin is produced on a mammalian cell obtainable according to the present invention. Said recombinant erythropoietin could be produced on PER.C6. The present invention further provides the use of PER.C6 for the production of a proteinaceous molecule comprising a predetermined post-translational modification, wherein it is preferred that said proteinaceous molecule is rapidly cleared from the blood and/or used in high dosage. In the case of EPO, produced on PER.C6, for example high dosage may be used to treat or prevent acute damage associated with hypoxia, while limiting the adverse side effects of erythropoiesis.

In one embodiment of the present invention, the proteinaceous molecules of the present invention are

suitable for the treatment of a human or a human body by surgery, therapy or diagnosis. Preferably said proteinaceous molecules such as EPO are used for the manufacture of a medicament for the treatment of hypoxia-induced disorders, neurodegenerative afflictions, or acute damage to the central- or peripheral nervous system. In another preferred embodiment, said proteinaceous molecules such as EPO are used for the manufacture of a medicament for the treatment of ischemia/reperfusion injuries. In yet another preferred embodiment, said proteinaceous molecules such as EPO are used for the manufacture of a medicament for the treatment of immune disorder and/or inflammatory disease.

Methods and compositions are disclosed herein for the production and manufacturing of recombinant proteins. The invention is particularly useful for the production of proteins that require co-translational and/or post-translational modifications such as glycosylation and proper folding and relates furthermore to the use of human cells capable of producing brain-type co- and/or post-translational modifications on proteinaceous molecules. These cells can for instance be used for the production of human glycoproteins with neural features that might be therapeutically beneficial, due to their neural features.

The present invention describes the use of a human cell line with neural characteristics that modifies recombinantly expressed proteins with neural properties such as 'brain-type' or 'neural-type' post-translational modifications such as glycosylation, phosphorylation or folding. An example of such a cell line, named PER.C6™ (U.S. Pat. No. 6,033,908), was generated by the immortalization of human embryonic retina cells using a

construct harboring the Adenovirus type 5 E1 genes. PER.C6 cells have proven to be particularly suitable for the production of recombinant human proteins, since high yields of proteins such as the human EPO and fully human monoclonal antibodies could be obtained (described in WO 00/63403). In general, the advantage of using PER.C6 for the production of recombinant proteins is the high level of production and the fact that the cells can be cultured either in an adherent fashion or in suspension in medium without serum or serum-derived components to very high densities.

The present invention discloses that PER.C6 cells express neural marker proteins, by showing that PER.C6 cells can be stained with specific antibodies against vimentin, synaptophysin, neurofilament, glial fibrillary acidic protein (GFAP), and neural cell adhesion molecules such as N-CAM and CD56. The presence of these marker proteins, as well as the morphological characteristics of the cells (e.g., an epithelial phenotype with large nuclei and many slender cytoplasmic processes well known to persons skilled in the art of histology) indicate that PER.C6 cells are of neural origin.

The invention further discloses that recombinant proteins produced by PER.C6 cells can acquire certain tissue specific features such as neural characteristics (e.g., post-translational modifications such as glycosylation). This is exemplified by the production of a protein that harbors so-called brain-type oligosaccharides. It is shown that human EPO produced by PER.C6 cells is modified with N-linked sugars that significantly differ from the N-linked sugars found in human urinary EPO or in recombinant human EPO produced by Chinese Hamster Ovary

(CHO) cells or Baby Hamster Kidney (BHK) cells. Human urinary EPO and recombinant human EPO produced in CHO and BHK cells contain glycosylation structures that can be referred to as 'renal-type' or 'serum-type' oligosaccharides. Typically, the N-linked sugars of these CHO- and BHK-EPO preparations are highly branched, highly galactosylated, and highly sialylated, whereas they lack peripheral α1,3-linked fucose (Tsuda et al. 1988; Takeuchi et al. 1988; Nimtz et al. 1993; Watson et al. 1994; Rahbek-Nielsen et al. 1997).

Herein, the nature of the oligosaccharides linked to human EPO produced on PER.C6 has been elucidated and shows that the oligosaccharides of PER.C6-produced human EPO differ significantly from the oligosaccharides present in human urinary EPO and recombinant human EPO produced in CHO and BHK cells. Firstly, the average sialic acid content of the oligosaccharides of PER.C6-produced human EPO is significantly lower than the average sialic acid content of human urinary EPO or recombinant human EPO (from CHO and BHK). Brain-type EPO does not circulate in the blood and it might very well be the reason that this EPO form does not need heavy sialylation for its protection against clearance. The very low sialic acid content in PER.C6-produced human EPO is indicative of the presence of N-linked oligosaccharides that contain terminating galactose and/or N-acetyl-galactosamine and/or N-acetyl-glucosamine. Secondly, N-acetyl-galactosamine is found in significant amounts in the N-linked sugars of PER.C6-produced human EPO, whereas N-acetyl-galactosamine is almost absent in the N-linked sugars of human urinary EPO and recombinant human EPO produced by CHO cells. Only trace amounts of N-acetyl-galactosamine have been reported to occur in the N-linked

sugars in a few batches of recombinant human EPO produced in BHK cells (Nimtz et al. 1993). Third, the N-linked sugars of human EPO produced in PER.C6 cells are found to contain a very high amount of fucose. A fraction of the fucoses is α1,3-linked to a peripheral N-acetyl-glucosamine thereby forming a so-called Lewis x structure (Fig. 5). Lewis x structures have never been reported to occur in human urinary EPO or in recombinant human EPO produced in CHO and BHK cells. Importantly, the (sialyl) Lewis x structures present on EPO might as a consequence relate to a role in EPO binding to selectins and a further role in cardioprotection. Such structures might be beneficial and perhaps indicative for a possible role for EPO in direct cardiac effects as discuused above. Taken together, the N-linked oligosaccharides present on PER.C6-produced human EPO have the strong characteristics of brain-type oligosaccharides.

Because the protein-linked oligosaccharides have a great impact on the physicochemical properties of the polypeptide such as tertiary conformation, solubility, viscosity, and charge, PER.C6-produced human EPO has physicochemical properties that differ significantly from human urinary EPO and recombinant human EPO produced by CHO and BHK cells (Toyoda et al. 2000). Clearly, PER.C6-produced human EPO is less charged than human urinary EPO and recombinant human EPO produced by CHO and BHK cells due to a lower sialic acid content and it is more hydrophobic due to the very high fucose content. As a result, the average pI of PER.C6-produced human EPO is significantly higher than the average pI of human urinary EPO or recombinant human EPO produced by CHO and BHK cells. Because the glycans of EPO, in particular the sialic acids,

also have an influence on the binding to the EPO receptor, it is expected that PER.C6-produced human EPO has a different affinity for the EPO receptor than human urinary EPO and recombinant human EPO produced by CHO and BHK cells.

The present invention furthermore discloses the use of brain-type proteins produced in neural human cells for the treatment of ischemia/reperfusion injury in mammals and especially in humans. Ischemia/reperfusion injury as used herein is defined as the cellular damage that occurs after reperfusion of previously viable ischemic tissues. Ischemia/reperfusion injury is associated with, for example, but not limited to thrombolytic therapy, coronary angioplasty, aortic cross clamping, cardiopulmonary bypass, organ or tissue transplantation, trauma and shock.

The present invention provides the use of therapeutic proteins, produced in mammalian cells, with brain-type oligosaccharides. These brain-type oligosaccharides comprise in particular Lewis x structures, sialyl Lewis x structures, or derivatives thereof containing the (sialyl) Lewis x structure, for the treatment of ischemia/reperfusion injury in mammalian subjects such as humans. The presence of (sialyl) Lewis x structures on recombinant proteins targets these proteins to the injured site of ischemia/reperfusion and thereby exerting their ischemia/reperfusion protective effect more effectively than proteins containing no (sialyl) Lewis x structures. The presence of brain-type oligosaccharides on recombinantly expressed proteins is exemplified in the present invention by Erythropoietin (EPO), which is produced on PER.C6 cells. This particular type of EPO contains the Lewis x as well as the sialyl Lewis x

structures. In the present invention experiments are described that show the superiority of PER.C6 brain-type (or neural-type) EPO compared to serum-type (or renal-type) EPO with respect to the cardioprotective function in *in vivo* models of cardiac ischemia/reperfusion injury.

An advantage provided by the present invention is that PER.C6-produced human EPO is less active in stimulating erythropoiesis *in vivo* than serum-type human EPO such as recombinant human EPO produced in CHO and BHK cells, which is currently used as a therapeutic drug to treat anemia in human beings. This means that when applied intravenously at the same dosis (i.e., equal amount of protein per kg body weight), PER.C6-produced human EPO causes a smaller increase in red blood cell production than the highly sialylated fraction of recombinant human EPO that is produced in CHO or BHK cells. As a result thereof, PER.C6-produced human EPO causes a smaller increase in the hematocrit value *in vivo* than the highly sialylated fraction of recombinant human EPO produced in CHO and BHK cells, when applied intravenously at the same dose. The poor effect of PER.C6-produced human EPO on erythropoiesis is most likely due to a relatively short half-life of the protein in the blood circulation, and/or due to an impaired targeting signal that directs the protein to erythroid progenitor cells in the bone marrow, and/or due to a low affinity of the protein for the EPO-R on erythroid progenitor cells. The impaired functioning of PER.C6-produced human EPO to stimulate erythropoiesis is a direct effect of its oligosaccharide composition, which is, as described above, significantly different from the oligosaccharide composition of serum-type EPO such as human

urinary EPO and recombinant human EPO produced in CHO and BHK cells.

Another important advantage presented by the present invention is that PER.C6-produced human EPO has a neurotrophic activity. PER.C6-produced EPO gives the EPO protein physicochemical and/or pharmacokinetic and/or pharmacodynamic advantages in functioning as a neurotrophic and/or neuro-protecting agent. PER.C6-produced EPO has higher affinity for neural cells and for the EPO-R on neural cells than the highly sialylated serum-type glycosylated human recombinant EPO produced in CHO and BHK cells. Recombinant human EPO produced on non-neural cells (Goto et al. 1988) has a lower affinity for the EPO-R on neural cells than for the EPO-R on erythroid progenitor cells (Musada et al. 1993 and 1994).

The neuroprotective role of EPO clearly opens new possibilities for the use of recombinant human EPO as neuroprotective therapy in response to toxic chemicals that may be induced by inflammation or by hypoxia and/or ischemia, or in neurodegenerative disorders. Yet, a major drawback is that when applied as a neuroprotective agent, recombinant EPO present in the blood circulation will also give rise to an increase of the red blood cells mass or hematocrit. This, in turn, leads to a higher blood viscosity, which may have detrimental effects in brain ischemia (Wiessner et al. 2001).

The present invention provides a solution for the problem that recombinant human EPO that has been applied thus far as a neuroprotective agent has the undesired haematotropic side effect (Wiessner et al. 2001). Thus, it is shown that PER.C6-produced brain-type glycosylated recombinant human EPO has a high potential as a

neurogenesis and/or a neuroprotective agent whereas it has a low potential in stimulating erythropoiesis.

According to the invention, PER.C6-produced EPO can be administered systemically (intra-venous, intra-peritoneal, intra-dermal) to inhibit, to prevent and/or to repair the neural damage that is caused by, for example, acute head and brain injury or neuro-degenerative disorders. The present invention also provides products that can be used to modulate the function of tissues that might get heavily damaged by hypoxia, such as the central- and peripheral nervous system, retinal tissue and heart tissue in mammals. Such tissues may be diseased but may also be normal and healthy. Disorders that can be treated by products provided by the present invention may result from acute head-, brain- and/or heart injuries, neuro-degenerative diseases, seizure disorders, neurotoxin poisoning, hypotension, cardiac arrest, radiation, multiple sclerosis and/or from injuries due to hypoxia. Hypoxia may be the result of prenatal- or postnatal oxygen deprivation, suffocation, emphysema, septic shock, cardiac arrest, choking, near drowning, sickle cell crisis, adult respiratory distress syndrome, dysrythmia, nitrogen narcosis, post-surgical cognitive dysfunction, carbon monoxide poisoning, smoke inhalation, chronic obstructive pulmonary disease anaphylactic shock or insulin shock. Seizure injuries include, but are not limited to, epilepsy, chronic seizure disorder or convulsions. In case the pathology is a result from neuro-degenerative diseases the disorder may be due to AIDS dementia, Alzheimer's disease, Parkinson's disease, Creutzfeldt-Jakob disease, stroke, cerebral palsy, spinal cord trauma, brain trauma, age-related loss of cognitive function, amyotrophic lateral sclerosis, alcoholism,

retinal ischemia, glaucoma, general neural loss, memory loss or aging. Other examples of diseases that may be treated with products provided by the present invention include autism, depression, anxiety disorders, mood disorders, attention deficit hyperactivity disorder (ADHD) and cognitive dysfunction.

PER.C6-EPO can passively cross the blood-brain barrier in case of blood-brain barrier dysfunction. In case the blood-brain barrier is intact, PER.C6-EPO might be actively transported over the blood-brain barrier through the EPO-R. Some studies suggested that EPO in itself is able to cross the blood-brain barrier when high doses of recombinant EPO is administered (WO 00/61164). Another possible route for recombinant PER.C6-EPO to cross the blood-brain barrier is via the interaction of the Lewis x glycan structures present on the PER.C6-produced EPO with E-selectin molecules present on human brain microvessel endothelial cells (Lou et al. 1996). Interaction between E-selectin and EPO may facilitate the transport of EPO across the cerebral endothelial barrier since E-selectin also has been implicated in the migration of T lymphocytes into the CNS (Wong et al. 1999). If required for optimal neuro-protection, PER.C6-produced EPO can be administered at a significantly higher dose than serum-type EPO, because PER.C6-EPO will induce erythropoiesis much less efficiently, such that the detrimental effects of the increase in hematocrit is reduced or even absent.

In another disclosure of the invention, PER.C6-EPO can be administered intrathecally by infusion, or through an indwelling ventricular catheter, or through lumbar injection, to inhibit or prevent neural damage. Again, the advantage of using brain-type EPO over serum-type EPO is

that in the event of leakage into the blood circulation in the case of blood-brain barrier dysfunction, due to for instance stroke, no undesired side-effects with respect to erythropoiesis will occur.

The present invention establishes that indefinitely growing transformed cells that grow to very high densities under serum-free conditions and that have strong neural characteristics, such as PER.C6, are extremely useful to produce factors that depend for their functionality on these characteristics. This inherently also provides the possibility to produce factors that do not have neural features or neural-related functions but that nevertheless benefit from the post-translational modifications that are brought about by such cells. One can envision that some factors also play a role in non-neural tissue but that still require glycosylation structures that include for instance Lewis x structures or fucose residues as described for EPO in the present invention and that can be provided by the means and methods of the present invention. Examples of factors that might be produced by PER.C6 and that take advantage of the neural characteristics of PER.C6 cells include, but are not limited to, brain-type erythropoietin, transferrin and the different factors mentioned above. The invention shows that it is very likely that the production of other recombinant neurotrophic glycoproteins will benefit from the brain-type modifications that take place in such cells.

In accordance with the present invention it has surprisingly been found, that erythropoietin-like molecules having on average a lower sialic acid residue count per protein backbone are still effective in the treatment

and/or prevention of various disorders. This opens entirely new ways to use EPO and EPO-like molecules hitherto believed to of less or no use, including but not limited to low-sialyl EPO-fractions of EPO batches produced on recombinant mammalian cell systems, discarded upon fractionation because of their low average sialylation degree and/or low associated erythropoietic activity. Thus, the present invention demonstrates that EPO with a low sialic acid content is about as potent in reducing infarct size in an experimentally induced stroke in rats as EPO with a higher sialic acid content. It is well established in the art that a high sialic acid content of EPO correlates to longer circulatory half-lifes and increased erythropoietic potential in vivo (Tsuda et al. 1990; Morimoto et al. 1996).

Hence, in general terms, the invention provides the use of a composition of erythropoietin-like molecules selected from the group consisting of one or more muteins of erythropoietin, one or more derivatives of erythropoietin, and a composition of one or more fractions of erythropoietin molecules sialylated to a varying degree, for the preparation of a medicament for the treatment of a disorder selected from the group consisting of ischemia, a reperfusion injury, a hypoxia-induced disorder, an inflammatory disease, a neurodegenerative disorder, and acute damage to the central- or peripheral nervous system, wherein said composition of erythropoietin-like molecules has on a protein content basis a lower erythropoietic activity *in vivo* than epoetin alfa. Embodiments of the invention comprise compositions and use thereof wherein said erythropoietic activity *in vivo* is at least 10, 20, 30, 40, 50, 60, 70, 80, or 90% lower than that of epoetin

alfa (Eprex). Erythropoietin-like molecules are meant to include molecules that have a protein backbone that is identical to or similar to the presently known forms of EPO, e.g. EPO muteins, EPO derivatives, or EPO molecules differing in glycosylation of the protein backbone in qualitative and/or quantitative respect. Muteins as used herein are meant to consist of erythropoietin-like molecules that have one or more mutations in the protein backbone by deletion, addition, substitution and/or translocation of amino acids relative to the protein backbone of epoietin alfa and shall include naturally occurring allelic variants as well as genetically and/or chemically and/or enzymatically obtained variants. Such molecules should still be able to confer a functional activity of EPO. They are obtainable using standard techniques of molecular biology, well known to those of skill in the art. A derivative as used herein is an erythropoietin-like molecule that is obtainable from erythropoietin or epoietin alfa, or any other functional mutein of epoietin alfa by the chemical or enzymatic modification thereof. Erythropoietic activity as meant herein is the stimulatory effect of EPO on red blood cell production in a human or animal subject, as can be measured by the increase in hematocrit values at a certain point in time after administration to the human or animal subject of erythropoietin-like molecules (e.g see example 9), or the measuring the hemoglobin concentration. These methods are all well known those of skill in the art. Epoetin alfa is the recombinant human EPO form present in currently marketed Eprex-TM, and is similar or identical (with respect to amino acid and carbohydrate composition) to human erythropoietin isolated from urine of anemic

patients. Treatment regimes for erythropoietic purposes are well established. In general EPO dosages are given in IU (international units), referring to the activity of EPO in erythropoiesis. Such IU correlate to the protein content of EPO but are operationally defined, and hence the correlation may vary between different batches. As a rule of thumb, one IU corresponds to 8-10 ng epoetin alfa. For the purpose of describing the invention the erythropoietic activity of the erythropoietin-like molecules is referred to on a protein content basis, to get rid of the variable introduced by defining IU. It will be clear to the person skilled in the art that although the IU are usually given for commercial EPO preparations, the concentration of EPO molecules in such preparations can easily be defined according to standard procedures. This will allow to determine the relative specific activity e.g in IU/g (see e.g. EP 0428267). Several *in vivo* and *in vitro* assays useful for these purposes are also described by Storring et al. (1992). Examples of other forms of EPO currently on the market are Procrit or Epogen (both epoetin alfa) and Aranesp (darbepoetin alfa, EPO with extra N-glycosylation sites to increase circulatory half-life and erythropoietic activity). Although the erythropoietic activity may vary somewhat between the various commercial epoetin alfa preparations on the market, they are generally optimized for high erythropoietic activity. The present invention discloses the use of EPO-like molecules or EPO-forms that have a lower hemopoietic or erythropoietic activity, thereby diminishing or avoiding the side-effects of increased erythropoiesis when this is not desired.

According to another embodiment of the invention, a composition of erythropoietin-like molecules is

characterized by an average number of sialic acid residues per erytropoetin-like molecule that is at least 10% lower than the average number of sialic acid residues per erythropoietin molecule in epoetin alfa. According to other embodiments, said average number of sialic acid residues may be chosen to be at least 20%, 30%, 40%, 50%, 60%, 70%, 80% or 90% lower than the average number of sialic acid residues per EPO protein backbone in epoetin alfa. Said average number of sialic acid residues in the erythropoietin-like molecule preferably lies between between 0 and 90% of the average number of sialic acid residues per EPO molecule in epoetin alfa, but the exact percentage may depend from disorder to disorder, and - sometimes - from patient to patient, as some patient - disorder combinations are less vulnerable to high hematocrit values than others. Alternatively, the number of sialic acid residues could be described per EPO-like molecule, e.g. 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, 1 or 0 sialic acid residues per EPO-like molecule. Since the values are averages calculated for a composition that consists of epo-like molecules of varying degree of sialylation, non-integer values in between the mentioned values are possible to define the molecules according to the invention. The optimal range could be determined empirically. The average number of sialic acid residues per molecule or the sialic acid content of EPO can be determined according to published procedures, and are well known to persons skilled in the art. One possible procedure is described in EP 0428267. In brief, the sialic acid residues are cleaved from the EPO-like molecules by hydrolysis with 0.35 M sulfuric acid at 80°C for 30 minutes, and the solutions are neutralized with sodium

hydroxide prior to analysis. Alternatively, the sialic acids can be removed by enzymatic cleavage according to standard procedures. The amount of EPO is estimated using well known procedures e.g. by using commercially available protein assay kits (e.g. Bradford assay, Biorad) and standard curves using recombinant human EPO as a standard, absorbance at 280 nm, ELISA, RIA, and the like. Sialic acid content can be analyzed by the procedure of Jourdian et al. (1971). Alternatively, sialic acids can be analysed using High Performance Anion-Exchange Chromatography, using procedures well known to the skilled person (e.g. Analysis of Sialic Acids using High-Performance Anion-Exchange Chromatography, Application note number TN41, Dionex). The sialic acid content can be expressed as moles of sialic acid per mole of EPO, or an average number of sialic acid residues per EPO-like molecule. An indication for the average number of sialic acid residues per EPO-like molecule can also be given by iso-electric focusing (see example 4), which measures the pI.

Several ways can be envisaged to obtain erythropoietin-like molecules with an average lower number of sialic acid residues per erythropoietin-like molecule. These include, but are not limited to treatment of EPO-like molecules, e.g.produced recombinantly in any suitable host cell line, using enzymes that cleave off the sialic acid in particular, such as neuraminidases, or enzymes that cleave off more substituents (including sialic acid) of the glycosylation structures, such as e.g. N-glycanase F (removes whole N-glycan), endoglycosidase F_2 (removes bi-antennary structures), endoglycosidase F_3 (removes bi- and tri-antennary structures, and the like, or treatment of EPO-like molecules with chemicals, including but not

limited to acids, that results in decrease of the average number of sialic acid residues per EPO-like molecule. In particular, a highly sialylated EPO fraction could be thus desialylated and used in the present invention. In yet another embodiment EPO-like molecules with an average lower number of sialic acid molecules are obtained by purifying or separating such forms from a mixture containing both higher and lower sialylated EPO. The currently used production systems generally result in such mixtures, and EPO that is intended for erythropoietic purposes is prepared by purifying the forms with a high average number of sialic acid residues. The present invention discloses use of other fractions from this process, i.e. the EPO forms with a lower number of sialic acid residues. Purifying or separating such fractions can be done using well-established techniques known to the skilled person, such as ion-exchange, affinity purification, and the like. The erythropoietin-like molecules of the invention are preferably produced recombinantly. This can be done in any suitable expression system, including but not limited to Chinese Hamster Ovary cells, Baby Hamster Kidney cells, human cells, such as HeLa, HEK293 or PER.C6. Expression in lower eukaryotic cells such as insect cells or yeast is also possible. Production of EPO-like molecules having low sialic acid content may be performed on sialylation-deficient cell systems, by way of a natural lack of sialylating enzymes, such as certain prokaryotic hosts, or by mutagenesis or genetic modification of hosts otherwise capable of producing sialylated proteins. Methods and means to produce recombinant proteins are well documented and known to the person skilled in the art, and it will be clear to the skilled person that using a different source

for the EPO-like protein is possible without departing from the scope of the invention. In one aspect of the invention, the EPO-like molecules are produced by methods according to the invention, thereby producing molecules with a predetermined post-translational modification.

In another aspect of the invention, the composition comprising erythropoietin-like molecules is characterized by the presence of erythropoietin-like molecules that once administered parenterally to a human or an animal subject are cleared from the bloodstream at a faster rate than epoetin alfa. Clearance from the bloodstream can be measured by methods well known in the art, e.g. by determining the half-life of a protein in blood such as done in example 18. In healthy volunteers epoetin alfa has a circulatory half-life of about 4 hours after repeated intravenous injections. A half-life of about 5 hours in patients with chronic renal insufficiency, and about 6 hours in children has been reported. Using the method of example 8, we measure a half-life of 180 min for epoetin alfa (Eprex). It should be clear to the skilled person that this method can be used to determine the half-life of the compositions of the invention, and express this half-life in hours or in a percentage of the half-life of the standard EPO (Eprex). Similar experiments are feasible in humans to determine the half-life in humans. Erythropoietin-like molecules with a lower ratio of tetra-antennary structures to bi-antennary structures will also have a shorter half life in plasma (Misaizu et al, 1995; Takeuchi et al, 1989). Production of EPO in cell lines that give rise to such lower ratios is feasible, or alternatively these forms are purified away from the forms containing more tetra-antennary structures. Such

compositions comprising relatively more bi-antennary structures are also useful according to the invention. It wil also be clear that one advantage of the current invention is that higher maximal concentrations of erythropoietin-like molecules in the circulation can be reached as compared to the currently used EPO forms such as Eprex, Procrit, NESP. If high concentrations of EPO-like molecules would be desired for said treatment, this can be done by administering high doses of the compositions of the invention. Administering of similar doses on a protein content basis of the currently used EPO-like molecules would lead to higher erythropoiesis, which is an undesired side-effect for said treatments.

The invention also provides pharmaceutical compositions comprising said erythropoietin-like molecules, and methods for treatment or preventing disorders selected from said groups, as wel as compositions of erythropoietin-like molecules for for the preventative and/or therapeutic treatment of the human or animal body.

EXAMPLES

Example 1. Studies on expression of marker proteins in PER.C6™ cells.

The cells that were transformed with the E1 region of human Adenovirus type 5 and that resulted in the PER.C6™ cell line (as deposited under ECACC no. 96022940) were derived from a human embryonic retina. Retinas generally comprise a number of different cells types (at least 55 different neural subtypes), including neural and fibroblast-like cells (Masland 2001). In order to trace the cellular origin of PER.C6, a study was performed to test the expression of marker proteins in or on the cells. These markers are known in the art to be characteristic for certain cell types and/or tissues. The marker proteins are given in Table I.

Marker protein expression was tested using antibodies directed against the marker proteins. In each experiment, a negative control (PER.C6 cells not incubated with antibody) and a positive control were taken along. These positive controls are sections of human tissue known to express the marker protein (Table II).

PER.C6 cells were cultured on glass slides in a medium chamber (Life Technologies, Nunc Lab-Tek, Chamber Slide, radiation sterilized, 2 medium chambers, cat.no. 154464A). PER.C6 cells were seeded at 65-70% confluency (2 wells per culturing chamber) and cultured for 24 h at 37°C (10% CO_2, 95% air). The medium was aspirated and the glass slides with cells were washed with sterile PBS, removed from the medium chamber and air-dried. Cells were fixed on the glass slides by incubation in acetone for 2 min. After air

drying, slides were wrapped in aluminum foil and frozen at a temperature lower than -18°C until use.

Positive control tissues were obtained from banks of tissue slides prepared for routine use at the division of pathology, Academic Hospital Erasmus University (Rotterdam, The Netherlands). Frozen sections were prepared (5 µm) and fixed in acetone, according to routine procedures.

The primary antibodies, their respective marker proteins, the suppliers and the catalog numbers of the antibodies are given in Table III. The dilutions, also detailed in Table III, are made in Phosphate Buffered Saline (PBS), 1% Bovine Serum Albumin. Incubations of the slides with the primary antibody were done for 30 min at room temperature, rinsed with PBS and incubated with the secondary antibody. These secondary antibodies were either goat anti rabbit (DAKO E0432; 1:50 dilution) or goat anti mouse (DAKO E0433; 1:50 dilution), depending on the nature of the primary antibody used. The second antibody was conjugated with biotin. After rinsing with PBS, the slides were incubated with streptavidin-avidin/biotin complex conjugated with alkaline phosphatase (DAKO, K0376). After 30 min of incubation, the samples were rinsed with Tris/HCl pH 8.0, developed with fuchsin substrate chromagen (DAKO K0624) in the dark room for 30 min. Subsequently, the slides were rinsed with tap water for 2 min and counterstained with hematoxylin according to routine procedures well known to persons skilled in the art. Then, the slides were examined microscopically and scored for marker protein expression (negative or positive). The results are presented in Table IV. For neurofilament staining (positive) not all PER.C6 cells did stain positive as a result of a different cell cycle- or maturation phase

of the cell population. This is a normal observation for neurofilament stainings.

From the data obtained it was concluded that PER.C6 cells are of neural origin since the cells stained positive for vimentin, synaptophysin, neurofilament, GFAP and N-CAM.

Example 2. Monosaccharide composition of PER.C6-EPO derived N-glycans compared to that of Eprex.

A first step in characterizing the N-glycan structures produced by PER.C6 is the measurement of the molar ratio of the various monosaccharides. The monosaccharide analysis was performed using high performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD). EPO samples, produced by PER.C6-derived clones P7, P8, and C25 (P7 and P8 are described in WO 00/63403, and C25 was generated generally according to these methods, using Neomycin resistance gene as a selection marker) in DMEM and/or JRH medium, were selected for this analysis. Eprex (Jansen Cilag), which is the commercially available recombinant CHO-derived erythropoietin, was analyzed in parallel, and therefore used as a reference.

PER.C6-EPO samples were purified by affinity chromatography using a column packed with C4 sepharose beads (bedvolume of 4 ml, Amersham Pharmacia Biotech) coupled with mouse monoclonal anti-EPO (IgG1) antibodies. Bound EPO molecules were eluted with 0.1 M glycine-HCl, pH 2.7, and resulting fractions were immediately neutralized by adding sodium/potassium phosphate buffer pH 8.0. Subsequently, the fractions containing EPO were pooled and the buffer was exchanged to 20 mM Tris-HCl, containing 0.1% (v/v) Tween 20, by utilizing Hiprep 26/10 desalting columns (Amersham Pharmacia Biotech).

For glycan analyses, purified EPO samples were dialyzed overnight against MilliQ-grade water, and dried in a Speedvac evaporator. Dried EPO samples (quantities ranged from 39 to 105 µg) were dissolved in incubation buffer (1:1 diluted C3 profiling buffer, Glyko). Upon addition of sodium dodecyl sulfate (SDS) and beta-mercaptoethanol to final concentrations of 0.1% (w/v) and 0.3% (v/v), respectively, samples were denatured for 5 min at 100°C. Nonidet P-40 (BDH) was thereafter added to a final concentration of 0.75% (v/v), and EPO was deglycosylated overnight at 37°C, using *N*-glycanase F (mU, Glyko). Upon deglycosylation, released N-glycans were separated from proteins, salts, and detergents by using graphitized carbon black (Carbograph) SPE columns (Alltech), according to Packer et al. (1998).

Purified N-glycan chains were subjected to hydrolysis in 2 M trifluoroacetic acid (TFA) at 100°C for 4 h. After hydrolysis, monosaccharides were dried in a Speedvac evaporator, washed with water, and again evaporated in a Speedvac. Dried monosaccharides were dissolved in 26 µl MilliQ-grade water. After addition of 6 µl deoxyglucose (100 nmol/ml), which was used as internal standard, samples (24.5 µl) were applied to an HPAEC-PAD BioLC system with a 2 mm-diameter CarboPac PA1 column (Dionex). The column was run isocratically in 16 mM NaOH (Baker) at a flow rate of 0.25 ml/min. The monosaccharide composition was calculated by comparing the profile with that obtained with a mixture of monosaccharide standards that consisted of fucose, deoxyglucose, galactosamine, glucosamine, galactose, and mannose.

The monosaccharide analysis clearly showed that the glycosylation status of PER.C6-EPO is significantly different from Eprex (Table V). The ratio of the indicated monosaccharides (Man = mannose, Fuc = fucose, GalNAc = N-acetyl-galactosamine, GlcNAc = N-acetyl-glucosamine, Gal = galactose) was normalized to 3 Man. The duplo values are given between brackets. Interestingly, the PER.C6-EPO samples contain significant amounts of GalNAc, whereas the N-linked sugars of Eprex lack this residue. This suggests that PER.C6-EPO contains so-called LacdiNAc (e.g., GalNAcβ1-4GlcNAc) structures. Another striking feature of PER.C6-EPO is the relative abundance of fucose residues shown in Table V. This strongly indicates the presence of Lewis structures in the N-glycans of PER.C6-EPO. In contrast, Eprex is known to be devoid of Lewis structures. Consequently, the amount of fucose found in Eprex can be solely attributed to N-glycan core fucosylation. Notably, the data from the monosaccharide analyses also demonstrated that culture conditions affect the glycosylation status of EPO in PER.C6. It should not be concluded that the culture conditions are solely responsible for the predetermined post-translational modifications that are present on the proteins produced. Of course the cell lines should be able to modify the post-translational modifications of the proteins produced on such cells through the presence of certain specific glycosylation enzymes such as transferases. The culture conditions can only exert additive activities. For instance, when the EPO-producing clones were cultured (in suspension) in JRH Excell 525 medium, the N-linked glycans of EPO were found to contain higher levels of GlcNAc, GalNAc, Gal, and Fuc as compared to the N-linked sugars of EPO derived from cultured

(adherent) cells in DMEM (Table V). This effect was particularly evident in the case of clone P8. The elevated level of GlcNAc may suggest that the branching of the N-linked sugars is increased and/or that the N-linked sugars contain more lactosamine repeats, when cells are cultured in JRH medium. The increase in N-acetyl glucosaminylation and in (N-acetyl-) galactosylation in turn gives rise to an increased number of fucose-acceptor sites thereby providing an explanation for the increase of the Fuc content.

Example 3. Mass spectrometric analysis to reveal structural differences between N-glycans of PER.C6-EPO and Eprex.

To obtain more detailed information on the structure of the N-glycans produced by PER.C6, it was decided to analyze the complete sugar chains of PER.C6-EPO by MALDI-MS. For this analysis, affinity-purified EPO samples, made by PER.C6-derived clones P7 and P8 in DMEM, which were fractionated further by anion exchange chromatography (as described below) were utilized. PER.C6-EPO samples, affinity-purified as described in example 2, of which the buffer was thereafter exchanged to PBS, were subjected to anion exchange chromatography using a HiTrap sepharose Q HP column (Amersham Pharmacia Biotech). Three EPO subfractions were obtained by applying a step gradient in 20 mM Tris-HCl/20 µM $CuSO_4$, beginning with 45 mM NaCl (fraction 1), followed by 75 mM NaCl (fraction 2), and ending with 135 mM NaCl (fraction 3). Each step of the gradient lasted 10 min with a flow rate of 1 ml/min. Fractions 1 of four runs were pooled into pool A, fractions 2 into pool B, and fractions 3 into pool C. The resulting pools A, B, and C were thereafter desalted utilizing HiPrep 26/10 desalting columns (Amersham Pharmacia Biotech). The N-linked glycans

were released from the EPO pools by N-glycanase F treatment and desialylated by neuraminidase treatment. Eprex was analyzed in parallel as a reference. Representative mass spectra of the various EPO samples are shown in Fig. 1A-G: Eprex and the purified, fractionated (pools A, B, and C from the anion exchange chromatography column). PER.C6-EPO samples derived from the indicated clones cultured in DMEM were treated with glycanase F and neuraminidase, and thereafter analyzed by MALDI-MS. Symbols (depicted in the spectrum of Eprex) are: closed square is GlcNAc, open circle is Man, closed circle is Gal, open triangle is Fuc. The mass profile of the N-linked sugars of Eprex (Fig. 1A) corresponds to previously published data and indicates that tetra-antennary sugars with or without lactosamine repeats predominate in this EPO preparation. Although Eprex and PER.C6-EPO contain sugar structures with a similar mass (Fig. 1B-G), the profile of the sugar structures of the latter is much more complex, suggesting that these sugars display a large degree of heterogeneity. The ExPAsy's computer program was used to predict the sugar composition on basis of the observed mass (Table VI and VII). The relative abundance of the different oligo-saccharides in each pool was also presented. Strikingly, the data demonstrated that most N-linked oligosaccharides derived from PER.C6-EPO contain multiple fucose residues (Table VI and VII, see level of dHex residues). Some glycans were even quadruple-fucosylated. Consequently, these data are in line with our monosaccharide analyses and strongly suggest that PER.C6-EPO is hyperfucosylated, and, hence, most likely decorated extensively with N-glycans having so-called Lewis structures. Oligosaccharides with (sialylated) Lewis x epitopes are known as essential recognition

sequences for selectins, mediating cell-cell adhesions in both inflammatory and immune responses (Varki et al. 1999) and are characteristically found in brain glycoproteins (Margolis and Margolis 1989). Hence, numerous glycoproteins carrying these Lewis x structures have been shown to have therapeutic potential by exhibiting anti-inflammatory and immunosuppressive activities. It is emphasized here that a mass signal cannot always be unambiguously assigned to a certain sugar structure, as e.g. residues, like GlcNAc and GalNAc, have the same mass. Because the monosaccharide analysis of PER.C6-EPO revealed the occurrence of GalNAc in the N-linked sugars, it is expected that some of the peaks represent N-glycans with so-called LacdiNAc (e.g., GalNAcβ1-4GlcNAc) structures. For example, peaks with *m/z* values of ~ 2038 and ~ 2185 (Table VI and VII) most likely represent N-glycans with LacdiNAc motifs. Otherwise, these peaks would represent tetra-antennary structures, which terminate in GlcNAc due to the absence of Gal or GalNAc. Although such structures may be present due to incomplete glycosylation, the presence of the proximal Fuc implies that the sugar contained a Gal or GalNAc residue that is necessary to form a motif that is recognized by the fucosyltransferase (FUT) that catalyzes the formation of the Lewis structure.

The relative occurrence of the different sugars varies between the EPO preparations derived from two independent PER.C6 clones as judged by the difference in the relative height of certain peaks. In particular, the putative bi-antennary sugars with LacdiNAc motifs (Fig. 1; Table VI and VII, signals with *m/z* values of ~ 2038 and ~ 2185) are clearly the major sugars in EPO samples derived from P8,

whereas in P7 samples these structures are far less abundant. In the latter clone, the peak with an *m/z* value of ~ 2541, putatively corresponding to a fully galactosylated tetra-antennary glycan, was the most abundant structure. These data are in accordance with our monosaccharide analyses, which already indicated that, when grown in DMEM, P8 produced EPO carrying glycans with a lower degree of branching than those derived from P7-EPO (Table V).

Example 4. Comparison of sialic acid content of PER.C6-EPO and CHO-EPO.

The sialic acid content of PER.C6-EPO was analyzed and compared with erythropoietin derived from Chinese Hamster Ovary cells (CHO-EPO) by iso-electric focusing (IEF) using IPG strips (Amersham Pharmacia Biotech) that have a linear pH gradient of 3-10. After the focusing, the EPO isoforms were passively blotted onto nitrocellulose, and visualized using an EPO-specific antibody and ECL (Fig. 2). EPO made by four different PER.C6 clones (lanes C, D, E, and F), and three different CHO clones stably expressing EPO (lanes G, H, and I) were analyzed by iso-electric focusing to determine the sialic acid content. The EPO producing CHO and PER.C6 cell lines were generated generally according to methods described in WO 00/63403 using the Neomycine-resistance gene as a selection marker. One thousand eU of PER.C6-EPO and 500 eU of CHO-EPO were loaded per strip. Five hundred IU of Eprex (lane A) and neuraminidase-treated (partially desialylated) Eprex (lane B) were used to identify the various EPO isoforms. After focusing, EPO was blotted onto nitrocellulose filter and visualized using a

monoclonal antibody against EPO and ECL. The Eprex sample, representing a commercially available EPO is a formulation containing highly sialylated isoforms and was used as a marker.

The results clearly demonstrated that CHO cells are able to make EPO isoforms containing up to at least 12 sialic acids per molecule (lanes G-I), confirming data by Morimoto et al. (1996). In contrast, although some isoforms with 8-10 sialic acids were produced by PER.C6, these were obviously underrepresented and only detectable after prolonged exposure of the film (lanes C-F). Consequently, it can be concluded that PER.C6-EPO is considerably less sialylated than CHO-EPO.

Example 5. α1,3-, α1,6- and α1,2-fucosyltransferase activities on PER.C6 cells.

The glycosylation potential of a cell is largely determined by an extensive repertoire of glycosyltransferases involved in the step-wise biosynthesis of N- and O-linked sugars. The activity of these glycosyltransferases varies between cell lines and, hence, glycoproteins produced in different cell lines acquire different glycans. In view of the data shown herein, demonstrating that PER.C6-EPO glycans are heavily fucosylated, the activity of numerous fucosyltransferases (FUTs) involved in the synthesis of N-linked sugars were analyzed using methods generally known to persons skilled in the art (Van den Nieuwenhof et al. 2000). In this study, we studied the activities of α1,6-FUT, which is involved in core fucosylation of N-glycans, α1,2-FUT which mediates the capping of terminal galactose residues, giving rise to so-called Lewis y epitopes, and α1,3-FUT, which generates

Lewis x structures. For comparison, we also analyzed the corresponding FUT activities present in CHO cells.

The activities of the indicated FUTs in cell-extracts of PER.C6 and CHO were measured using a glycosyltransferase activity assay. This assay measures the glycosyltransferase-catalyzed reaction between a saccharide (in this case fucose) and a sugar substrate. The GalT activity was also measured as an internal control. The values represent the mean values from two experiments. All values, and in particular those of PER.C6 were 2-3 fold lower in the second experiment. Notably, the activities were expressed per mg protein (present in the cell extract). Because PER.C6 cells are significantly bigger than CHO cells, the differences between the FUT and GalT activities of CHO and PER.C6 cells may be bigger or smaller than they appear. The results of the glycosyltransferase activity assays are shown in Table VIII and reveal that PER.C6 as well as CHO possess significant α1,6-FUT activity, which suggests that both cell lines can produce core-fucosylated glycan chains. α1,3-FUT activity was, however, only significant in PER.C6 cells while hardly detectable in CHO cells. None of the two cell lines exhibited α1,2-FUT activity. Taken together, these data show a clear difference between the glycosylation potential of CHO and PER.C6, and explain why PER.C6-EPO contains more fucoses than CHO-produced EPO (Eprex).

Example 6. Glycans with Lewis x epitopes present on PER.C6-EPO.

Because PER.C6 possesses α1,3-, but no α1,2-fucosyltransferase activity, it is very likely that PER.C6 produced N-glycan chains which contain Lewis x instead of

Lewis y epitopes. We verified this by labeling PER.C6-EPO with a mouse monoclonal antibody (Calbiochem) that specifically recognizes Lewis x structures, using western blotting. Equal amounts of PER.C6-EPO (derived from clone P7, here indicated as P7.100) and Eprex, untreated (-) or treated with HCl (+), were run on a SDS-polyacrylamide gel and blotted onto a nitrocellulose membrane using methods known to persons skilled in the art. A monoclonal antibody (anti-mouse IgM, Calbiochem) and ECL (Amersham Pharmacia Biotech) were used to detect the Lewis x epitope. As can be seen in Fig. 3, only PER.C6-EPO could be labeled with the antibody specific for the Lewis x epitope. Location of the molecular weight marker (52, 35 and 29 kDa) is indicated. Because the α1,3-fucose linkage is acid-labile, the signal was lost after treatment with HCl. Yet, it has to be noted that the acid treatment also destroyed part of the EPO since a reduced signal was observed when the blot was probed with an EPO-specific antibody (not shown).

Example 7. Lewis x structures expression at cell surface of PER.C6 cells.

To find out whether Lewis x structures generally occur in PER.C6 cells, we labeled the surface of CHO and normal (i.e., not EPO producing) PER.C6 cells with Lewis x specific antibodies (Calbiochem). The cells were incubated with the primary antibodies (mAb α Lewis x used at 0.16 µg/ml, and mAb α sialyl-Lewis x used at 5 µg/ml). FITC-conjugated anti-IgM was used as a secondary antibody. The labeled cells were analyzed by FACS. The dashed line represents the signal of cells incubated with the secondary antibody only (negative control). The results shown in Fig. 4 revealed that PER.C6 cells were strongly labeled with the

antibodies in contrast to CHO cells that are unable to produce these structures. Notably, we repeatedly observed that PER.C6 cells displayed a heterogeneous pattern of staining with the Lewis x antibodies. Labeling with an antibody specific for sialyl Lewis x structures (Calbiochem) gave a moderate positive signal only when a very high concentration of the antibody was used.

Example 8. Inhibition of apoptosis by PER.C6-EPO (brain-type) *in vitro*, in NT2 cells and hNT cells cultured under hypoxic conditions.

PER.C6-produced (brain-type) EPO and serum-type EPO are compared in their *in vitro* activity to protect rat-, mouse- and human cortical neural cells from cell death under hypoxic conditions and with glucose deprivation. For this, neural cell cultures are prepared from rat embryos as described by others (Koretz et al. 1994; Nagayama et al. 1999; White et al. 1996). To evaluate the effects of PER.C6-produced brain-type EPO and serum-type EPO, the cells are maintained in modular incubator chambers in a water-jacketed incubator for up to 48 h at 37°C, in serum-free medium with 30 mM glucose and humidified 95% air/5% CO_2 (normoxia) or in serum-free medium without glucose and humidified 95% N_2/5% CO_2 (hypoxia and glucose deprivation), in the absence or presence of 30 pM purified PER.C6-produced brain-type EPO or 30 pM Eprex. The cell cultures are exposed to hypoxia and glucose deprivation for less than 24 h and thereafter returned to normoxic conditions for the remainder of 24 h. The cytotoxity is analyzed by the fluorescence of Alamar blue, which reports cells viability as a function of metabolic activity.

In another method, the neural cell cultures are exposed for 24 h to 1 mM L-glutamate or α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) under normoxic conditions, in the absence or presence of various concentrations of purified PER.C6-produced EPO or Eprex. The cytotoxity is analyzed by the fluorescence of Alamar blue, which reports cell-viability as a function of metabolic activity. The viability of cells treated with PER.C6-EPO is expected to be similar to the viability of cells treated with Eprex.

Example 9. Activity of PER.C6-EPO (brain-type) in stimulating erythropoiesis in rats compared to serum-type EPO.

The potential of recombinant human EPO to stimulate the production of red blood cells can be monitored in a rodent model that has been described by Barbone et al. (1994). According to this model, the increase in the reticulocyte counts is used as a measure for the biological activity of the recombinant human EPO preparation. Reticulocytes are the precursors of red blood cells and their production, in response to EPO, can be used as a measure for the potential of EPO in stimulating the production of red blood cells. An increased production of red blood cells, in turn, leads to a higher hematocrit value.

The activities of PER.C6-EPO and Eprex were compared in six groups of three Wag/Rij rats. Various doses of PER.C6-EPO (P7-EPO), Eprex and diluent buffer as a negative control were injected intravenously in the penile vein at day 0, 1, and 2. PER.C6-EPO was administered at a dose of 5, 25, or 125 eU (Elisa units) as determined by the

commercially available EPO-specific R&D Elisa Kit, whereas Eprex was administered at a dose of 1 or 5 eU. All EPO preparations were diluted to the proper concentration in PBS/0.05% Tween 80 in a total volume of 500 µl. At day 3, 250 µl of EDTA blood was sampled by tongue puncture. On the same day, the percentage of reticulocytes in the total red blood cell population was determined.

As shown in Fig. 6 (bars indicate the percentage of reticulocytes present in the total red blood cell population), the daily administration of 1 eU of Eprex into the rats, for a total period of three days, caused a significant increase in the reticulocyte counts at the fourth day compared to reticulocyte counts in rats that received diluent buffer only. The reticulocyte counts were even more boosted by increasing the Eprex dose five-fold. The reticulocyte counts were clearly less increased using equivalent amounts of PER.C6-EPO. A similar increase in reticulocyte counts was observed when 1 eU of Eprex and 25 eU of PER.C6-EPO was used indicating that PER.C6-EPO is at least 25 times less active in stimulating the red blood cell production than Eprex. The difference between the potential of Eprex and PER.C6-EPO in stimulating the red blood cell production was even more pronounced at a higher dose (i.e. 5 eU Eprex and 125 eU PER.C6-EPO).

Example 10. Effect of PER.C6-EPO on cerebral ischemia following experiment subarachnoid hemorrhage.

To show that PER.C6-EPO is more effective in neuro-protection during cerebral ischemia than serum-type EPO, we compare the effects of systemic adminstration of PER.C6-produced brain-type EPO and serum-type EPO in a rabbit model of subarachnoid hemorrhage-induced acute cerebral

ischemia. Therefore, 32 animals that are divided into 4 groups (n=8) are studied.

Group 1, subarachnoid hemorrhage;
Group 2, subarachnoid hemorrhage plus placebo;
Group 3, subarachnoid hemorrhage plus recombinant human serum-type EPO; and
Group 4, subarachnoid hemorrhage plus recombinant PER.C6-produced EPO.

The experimental subarachnoid hemorrhage is produced by a percutaneous injection of autologous blood into the cisterna magna after anesthesizing the animal. After the injection, the rabbits are positioned in ventral recumbence for 15 min to allow ventral blood-clot formation. Animals of group 2, 3, and 4 are injected with diluent buffer, Eprex, and purified PER.C6-produced brain-type EPO, respectively, at 5 min after the induction of subarachnoid hemorrhage, and are continued at 8, 16, and 24 h thereafter. All injections are administered intra-peritoneally. The diluent buffer consists of serum albumin (2.5 mg/ml), sodium chloride (5.84 mg/ml), anhydrous citric acid (0.057 mg/ml, H_2O). The animals are euthanized at 24 h after the subarachnoid hemorrhage, and their brains are removed. The brains are thereafter coronally sectioned at 10-25 µm in a freezing microtome, starting at the bregma and continuing posteriorly to include the cerebellum (Ireland and MacLeod 1993). To visualize and assess the number of ischemia-induced damaged neurons, the slices are stained with hematoxylin and eosin. The number of eosinophilic neuronal profiles containing pyknotic nuclei, per high-power microscopic field (100x) is determined in

five randomly selected sections of the lateral cortex obtained at several coronal levels posterior to the bregma. PER.C6-EPO treated animals are expected to have a lower number of damaged neurons than animals that are not treated or that are treated with a placebo.

Example 11. Erythropoietin receptor expression in rat neonatal cardiomyocytes following hypoxia/reoxygenation.

Primary cultures of neonatal rat cardiomyocytes are prepared from the ventricles of 1-day-old Sprague-Dawley rats, as previously described (Simpson and Savion 1982). Hypoxia was created by incubating the cardiomyoctes in an airtight Plexiglas chamber with < 1% O_2 and 5% CO_2/95% N_2 at 37°C for 2 h using Gas Pak Plus (BBL). By replacing the medium saturated with 95% air and 5% CO_2, the cells were exposed to normotoxic atmosphere (reoxygenation).

Cardiomyoctes are washed twice with ice-cold PBS and total RNA is isolated using Trizol (GIBCO), extracted by chloroform and precipitated by isopropyl alcohol. For Northern analysis, 15 µg of total RNA is separated on a 1.5% formaldehyde/MOPS-agarose gel, blotted to nitrocellulose, and hybridized with a ^{32}P-labeled probe for EPO receptor (± 400 bp cDNA fragment). Hybridization takes place overnight at 65°C in phosphate buffer, pH 7.2 and is followed by 2 washes in 2xSSC at room temperature, 2 washes in 0.2xSSC/0.1%SDS at 65°C and 2 washes in 2xSSC at room temperature. Hybridization signals are visualized by exposing the membrane to an X-ray film (Kodak). Expression levels are corrected for GAPDH mRNA levels.

Example 12. The effect of brain-type PER.C6-EPO and serum-type EPO (Eprex) on apoptosis in rat neonatal cardiomyocytes, cultured under hypoxic conditions.

Primary cultures cultures of neonatal rat cardiomyocytes are prepared from the ventricles of 1-day-old Sprague-Dawley rats as previously described (Simpson and Savion 1982). Hypoxia is created by incubating the cardiomyoctes in an airtight Plexiglas chamber with < 1% O_2 and 5% CO_2/95% N_2 at 37°C for 2 h using Gas Pak Plus (BBL). By replacing the medium saturated with 95% air and 5% CO_2, the cells are exposed to normotoxic atmosphere (reoxygenation). The experiment is divided into 4 groups:

A) cardiomyocytes cultured under normoxic conditions (95% air/5% CO_2);

B) cardiomyocytes cultured under hypoxia/reoxygenation conditions in the presence of 30 pM purified PER.C6-produced EPO;

C) cardiomyocytes cultured under hypoxia/reoxygenation conditions in the presence of 30 pM purified Eprex; and

D) cardiomyocytes cultured under hypoxia/reoxygenation conditions in the absence of EPO.

All experiments are performed in triplicate. Apoptosis is quantified by morphological analysis, DNA laddering and by terminal deoxyribonucleotide transferase-mediated dUTP nick end labeling (TUNEL). For morphological analysis myocytes monolayers are fixed and stained with Hoechst 33324. The morphological features of apoptosis (cell shrinkage, chromatin condensation, and fragmentation) are monitored by fluorescence microscopy. At least 400 cells from 12 randomly selected fields per dish are counted.

For determining DNA laddering (characteristic for apoptosis), cardiomyocytes are lysed in lysis buffer and electrophoresed on 2% agarose gel. The gel is stained with ethidium bromide, and DNA fragments are visualized under ultraviolet light. *In situ* detection of apoptotic cardiomyocytes is performed by using TUNEL with an in situ cell death detection kit (Boehringer Mannheim).

Example 13. The effect of PER.C6-EPO and serum-EPO on the infarct size in a rat model of myocardial ischemia/ reperfusion.

Adult male Sprague-Dawley rats (300 to 400 g) are anesthetized with sodium pentobarbital (20 mg/kg IP) and ketamine HCl (60 mg/kg IP). Jugular vein and trachea are cannulated, and ventilation is maintained with 100% oxygen by a rodent ventilator adjusted to maintain exhaled CO_2 between 3.5% and 5%. A left thoracotomy was performed and a suture was placed 3 to 4 mm from the origin of the left coronary artery. Five minutes before ischemia animals are randomly given various concentrations of PER.C6-EPO, serum-type EPO or saline (n=6 for each group). Ischemia (30 min) is initiated by tightening of the suture around the coronary artery and is followed by 4 h of reperfusion. Sham-operated rats are prepared identically, except that the suture is not tightened (n=6).

After reperfusion, infarct size is determined by differential staining with patent blue violet (5%) and triphenyl tetrazolium chloride (TTC). The coronary ligature is retightened, and an intravenous injection of patent blue violet is given to stain the normally perfused regions of the heart. The heart is then removed and bathed in ice-cold saline before removal of the atria, great vessels and right

ventricle. The left ventricle is sliced into thin sections, and the unstained area at risk (AAR) is separated from the normally perfused blue sections, cut into 1-2 mm^3 pieces, and incubated with TTC. With a dissecting microscope, the necrotic areas (AN, pale) are separated from the TTC-positive (brick red-staining) areas. All areas of the myocardium are then weighed individually, and infarct size is calculated.

Example 14. Isolation and fractionation of PER.C6-EPO glycoforms employing a high α1,3-linked fucose content.

The fucose-specific *Aleuria aurantia* lectin (AAL) is used to preferentially purify PER.C6-EPO glycoforms with a high Lewis x and/or sialyl-Lewis x content. This lectin is coupled to CNBr-activated Sepharose 4B beads according to procedures commonly known by a person skilled in the art. PER.C6-EPO that is secreted into the culture medium by human EPO-producing PER.C6 cells is first roughly separated from cell debris and other contaminants by affinity column chromatography using monoclonal antibodies specific for human EPO. Thereafter, the purified EPO is subjected to a second chromatography procedure in which the EPO molecules possessing α1,3-linked fucose are bound to a column containing the immobilized AAL. EPO glycoforms that lack α1,3-linked fucose do not bind to the column and are collected in the flow-through. EPO glycoforms carrying α1,3-linked fucose are eluted from the column by using fucose as a competitor for binding to AAL. EPO glycoforms having a high or low α1,3-linked fucose content are separately eluted from the column by increasing the fucose concentration step-wise or gradually during the elution.

EPO glycoforms with a high α1,3-linked fucose content are eluted at a higher concentration of fucose than EPO glycoforms with a low α1,3-linked fucose content. This method enables one to purify erythropoietin from the culture medium by employing the specific characteristics of the post-translational modifications, such as Lewis x structures brought about by the cells in which the protein is produced.

Example 15. Isolation and fractionation of PER.C6-EPO glycoforms with a high LacdiNAc content.

PER.C6-EPO glycoforms carrying so-called lacdiNAc oligosaccharide structures are specifically isolated by the use of monoclonal antibodies against these lacdiNAc structures. Mouse monoclonal antibodies such as 99-2A5-B, 100-2H5-A, 114-2H12-C, 259-2A1, and 273-3F2 (Van Remoortere et al. 2000) specifically recognize lacdiNAc structures and are purified and coupled to CNBr-activated Sepharose 4B beads according to procedures commonly known by a person skilled in the art. PER.C6-EPO that is secreted into the culture medium by human EPO-producing PER.C6 cells is first roughly separated from cell debris and other contaminants by affinity column chromatography using monoclonal antibodies specific for human EPO. Thereafter, the purified EPO is subjected to a second chromatography procedure in which the EPO molecules carrying lacdiNAc structures are bound to a column containing the immobilized lacdiNAc-specific monoclonal antibodies. EPO glycoforms that lack the lacdiNAc structures do not bind to the column and are collected in the flow-through. EPO glycoforms carrying the lacdiNAc structures are eluted from the column at a low pH or by using GalNAc or synthetic lacdiNAc oligosaccharides

as a competitor for binding to the lacdiNAc specific antibodies. EPO glycoforms carrying a relatively high percentage of lacdiNAc structures are separately eluted from the column by increasing the GalNAc or lacdiNAc concentration step-wise or gradually during the elution. EPO glycoforms with a relatively high percentage of lacdiNAc structures are eluted at a higher concentration of GalNAc or lacdiNAc than EPO glycoforms possessing a relatively low percentage of lacdiNac structures. In accordance with the method described above, also this method enables one to purify erythropoietin from the culture medium by employing the specific characteristics of the post-translational modifications, such as Lewis x and lacdiNAc structures brought about by the cells in which the protein is produced.

Example 16. Isolation and fractionation of PER.C6-EPO glycoforms with a high GalNAc-Lewis x content.

PER.C6-EPO glycoforms carrying so-called GalNAc-Lewis x oligosaccharide structures are specifically isolated by the use of monoclonal antibodies against these GalNAc-Lewis x structures. Mouse monoclonal antibodies such as 114-5B1-A, 176-3A7, 290-2D9-A, and 290-4A8 (Van Remoortere et al. 2000) specifically recognize GalNAc-Lewis x structures and are purified and coupled to CNBr-activated Sepharose 4B beads according to procedures commonly known by persons skilled in the art. PER.C6-EPO that is secreted into the culture medium by human EPO-producing PER.C6 cells is first roughly separated from cell debris and other contaminants by affinity column chromatography using monoclonal antibodies specific for human EPO. Thereafter, the purified EPO is subjected to a second chromatography procedure in

which the EPO molecules carrying GalNAc-Lewis x structures are bound to a column containing the immobilized GalNAc-Lewis x specific monoclonal antibodies. EPO glycoforms that lack the GalNAc-Lewis x structures do not bind to the antibodies attached to the column and are collected in the flow-through. Bound EPO glycoforms carrying the GalNAc-Lewis x structures are eluted from the column at low pH or by using synthetic GalNAc-Lewis x as a competitor for binding to the GalNAc-Lewis x specific antibodies. EPO glycoforms carrying a high GalNAc-Lewis x content can be separately eluted from the column by increasing the concentration of GalNAc-Lewis x competitor step-wise or gradually during the elution. EPO glycoforms with a high GalNAc-Lewis x content are eluted at a higher concentration of GalNAc-Lewis x than EPO glycoforms possessing a low GalNAc-Lewis x content. Again, in accordance with the methods described above, also this method enables one to purify EPO from the culture medium by employing the specific characteristics of the post-translational modifications, such as Lewis x, lacdiNac or GalNac-Lewis x structures brought about by the cells in which the protein is produced. This does however, not imply that other modifications with the (predetermined) post-translational modifications cannot be employed for proper purification of the protein.

It will be understood by those of skill in the art, that although the invention has been illustrated with detailed examples concerning EPO, the present invention is not limited to production and/or purification of EPO with brain-type characteristics. Various other (human) therapeutic and/or diagnostic peptides and proteins, which

may find use in treating disorders of the brain and other parts of the central- and peripheral nervous system and/or other ischemic/reperfusion damaged tissues, can be produced by means and methods of the present invention.

Example 17. EPO with a low sialic acid content has a similar potency as EPO with a high sialic acid content in reducing the infarct size after middle cerebral artery occlusion in rats.

The effect of PER.C6-EPO and Eprex on the size of a brain infarct, which was experimentally induced by the occlusion of the middle cerebral artery (MCA), was studied in Wistar male rats weighing 200-250g, using a method similar to the method published by Sirén et al., 2001. The right carotid artery of the animals was permanently occluded whereas the MCA was reversibly occluded for 60 min using a metal clip. Purified PER.C6-EPO with an average sialic acid content of < 6 sialic acids per molecule or Eprex (Jansen-Cilag; commercially available EPO) with an average sialic acid content > 9 sialic acids per molecule) was applied intravenously at 5 min before the onset of the MCA occlusion at a dose of 5000 eU (ELISA units) per kg body weight. Notably, the sialic acid content of the PER.C6-EPO preparation ranged from 0-9 sialic acids per molecule whereas Eprex contained more than 8 sialic acids per molecule. After a 60-min period, the occlusion was terminated by the removal of the metal clip surrounding the MCA. Reperfusion was observed microscopically after the removal of the clip. Twenty-four hr later the brains of the living rats were examined using MRI to reveal the Apparent Diffusion Coefficient (ADC) and T2 maps. These maps were used to quantify the infarct volumes (Figs. 7A and 7B).

The results in Figs 7A and 7B show that rats treated with the PER.C6-EPO and Eprex preparations displayed a similar reduction in the infarct size compared to the non-treated animals. Since the PER.C6-EPO preparation has a much lower sialic acid content than the Eprex preparation this result demonstrates that a high sialic content is not essential for the neuroprotective activity of EPO in vivo.

Example 18. Determination of half-life of EPO in rats.

To determine the half-life of Eprex in vivo, male Wag/Rij rats have been injected intravenously with 150 eU Eprex diluted in PBS/0.05% Tween-80 to a final volume of 500 µl. Just before the administration of the substrate, 200 µl of EDTA blood was sampled as negative control using the technique described in Lab. Animals 34, 372. At t=5, 15, 30, 60, 120, 180, 240, 300, 360, 420, 480, and 540 min after injection 200 µl EDTA blood was taken from the animals using the same technique. After the last blood sampling, the animals were sacrificed. The specimen was centrifuged at 760 x g for 15 min at RT within 30 min of collection. The plasma samples were tested in an EPO specific Elisa (R&D) to determine the concentration of EPO in each sample.

As shown in Fig. 8, the decrease in the concentration of Eprex in the plasma displays a bi-phasic curve representing a distribution phase and a clearance phase. On basis of these results it can be estimated that Eprex had a half-life of about 180 min during the clearance phase. The

half-life of PER.C6-EPO is measured using the same protocol.

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Table I.

Marker protein	Description
Pan-keratin	Detection of almost all cytokeratins. Labels keratinized and corneal epidermis, stratified squamous epithelia of internal organs, stratified epithelia, hyperproliferative keratinocytes, and simple epithelia.
EMA	Epithelial membrane antigen. Labels normal and neoplastic epithelium.
S100	EF-band type Ca^{2+} binding proteins. Expressed in neural tissues and other tissues.
Vimentin	Cytoskeletal intermediate filaments (= structural protein). Is a general marker of cells originating in the mesenchyme. Expressed during skeletal muscle development.
Desmin	Cytoskeletal intermediate filaments (= structural protein). Expressed during skeletal muscle development.
s.m. actin	Smooth muscle cell actin. Stains smooth muscle cells and myo-epithelial cells.
Synaptophysin	Reacts with neuroendocrine cells.
Chromogranin	Acidic glycoproteins that are widely expressed within secretory granules of endocrine, neuroendocrine and neural tissue.
NSE	Neuron specific enolase. Labels cells of neural and neuroendocrine origin
Neurofilament	Reacts with phosphorylated neurofilament protein and Labels neural processes and pheripheral nerves as well as symphataetic ganglion cells and adrenal medulla.
GFAP (polycon)	Glial Fibrillary Acidic Protein. GFAP is specifically found in astroglia, which are highly responsive to neurologic insults. Astrogliosis is found to be a result of mechanical trauma, AIDS dementia and prion infection and is accompanied by an increase in GFAP expression. Immunohistochemical marker for localizing benign astrocyte and neoplastic cells of glial origin in the central nervous system.
CD31	Reacts with PECAM-1. Present on platelets, monocytes, granulocytes, lymphocytes, endothelial cells.
CD34	Recognizes O-glycosylated transmembrane glycoprotein. Expressed on hemopoietic stem cells, vascular EC, embryonic fibroblasts, some cells in fetal adult nerve tissue.
N-CAM	Neuronal cell adhesion molecules. N-CAM is involved in cell-cell interactions during growth.

Table II.

Marker protein	Control tissue
Pan-keratin	Colon carcinoma
EMA	Colon carcinoma
S100	Pancreas
Vimentin	Tonsil
Desmin	Colon
s.m. actin	Tonsil
Synaptophysin	Pancreas
Chromogranin	Pancreas
NSE	Pancreas
Neurofilament	Colon
GFAP (polycon)	Brain
CD31	Colon
CD34	Tonsil
N-CAM (CD56)	Colon

Table III.

Marker protein	Supplier	Antibody	Catalog nr	Antibody dilution
Pan-keratin	Biogenex	Mouse IgG1	MU071-UC	1:200
EMA	Dako	Mouse IgG2a	M0613	1:50
S100	Dako	Rabbit	Z0311	1:3000
Vimentin	Biogenex	Mouse IgG1	MU074-UC	1:3200
Desmin	Sanbio	Mouse IgG	MON 3001	1:50
s.m. actin	Biogenex	Mouse IgG2a	MU128-UC	1:150
Synaptophysin	Dako	Mouse IgG1	M0776	1:50
Chromogranin	Biogenex	Mouse IgG1	MU126-UC	1:150
NSE	Dako	Mouse IgG1	M0873	1:250
Neurofilament	Sanbio	Mouse IgG	MON3004	1:300
GFAP (polycon)	Dako	Mouse IgG1	M0761	1:200
CD31	Dako	Mouse IgG1	M0823	1:60
CD34	Biogenex	Mouse IgG1	MU236-UC	1:20
N-CAM (CD56)	Neomarkers	Mouse IgG1	MS.204.P	1:10

Table IV.

Marker protein	Score
Pan-keratin	Negative
EMA	Negative
S100	Negative
Vimentin	**Positive**
Desmin	Negative
s.m. actin	Negative
Synaptophysin	**Positive**
Chromogranin	Negative
NSE	Negative
Neurofilament	**Positive**
GFAP (polycon)	**Positive**
CD31	Negative
CD34	Negative
N-CAM (CD56)	**Positive**

Table V.

Clone and culture conditions	Molar ratio of neutral monosaccharides normalized to three mannose residues				
	Man	**Fuc**	**GalNAc**	**GlcNAc**	**Gal**
P8 - DMEM	3	0.5 (0.9)	0.4 (0.4)	2.2 (2.7)	1.7 (1.3)
P8 - JRH	3	1.5 (1.4)	0.7 (0.8)	6.1 (6.4)	3.5 (3.9)
P7 - DMEM	3	1.5 (1.4)	0.4 (0.3)	5.5 (6.1)	2.3 (3.3)
P7 - JRH	3	1.8 (1.7)	0.4 (0.4)	6.1 (6.8)	3.6 (4.2)
C25 - DMEM	3	2.0	1.0	6.0	2.2
Eprex	3	0.7	-	5.4	4.1

Table VI.

P7	Percentage of total			Ratio
Mass (m/z)	Pool A	Pool B	Pool C	Hex:HexNAc:dHex
1809.64	2.34	2.99	2.44	5:4:1
1850.67	2.57	5.31	2.49	4:5:1
1891.69	5.06	10.39	1.31	3:6:1
1955.70	-	1.95	2.16	5:4:2
1996.72	6.37	7.96	6.38	4:5:2
2037.75	6.33	5.16	5.39	3:6:2
2053.74	3.70	4.11	1.98	5:5:1
2142.78	2.19	3.68	2.45	4:5:3
2174.77	6.53	3.63	8.04	6:5:1
2183.81	6.69	5.02	7.57	3:6:3
2199.80	3.78	4.65	1.58	4:6:2
2215.80	4.13	4.95	4.15	5:6:1
2256.82	-	1.30	-	4:7:1
2320.83	2.34	2.04	3.29	6:5:2
2361.86	4.35	3.30	3.23	5:6:2
2377.85	3.77	3.79	2.86	6:6:1
2507.91	1.62	2.32	1.32	5:6:3
2523.91	2.09	2.60	1.61	6:6:2
2539.90	11.89	4.81	19.32	7:6:1
2580.93	3.32	1.53	1.69	6:7:1
2612.94	-	-	1.78	6:5:3
2669.97	1.95	2.34	-	6:6:3
2685.96	6.21	3.11	5.81	7:6:2
2726.99	1.62	1.38	1.36	6:7:2
2832.02	3.64	1.55	3.08	7:6:3
2905.04	1.79	-	2.45	8:7:1
2978.08	2.23	1.65	-	7:6:4

Table VII.

P8	Percentage of total			Ratio
Mass (m/z)	Pool A	Pool B	Pool C	Hex:HexNAc:dHex
1809.64	-	1.03	-	5:4:1
1850.67	3.36	2.05	-	4:5:1
1891.69	5.11	2.11	3.04	3:6:1
1955.70	1.46	1.22	1.08	5:4:2
1996.72	5.05	4.61	6.54	4:5:2
2012.72	1.34	1.38	1.35	5:5:1
2037.75	14.62	14.34	12.48	3:6:2
2053.74	3.73	2.76	4.29	4:6:1
2142.78	2.57	1.97	2.06	4:5:3
2158.78	1.43	1.91	-	5:5:2
2174.77	2.40	2.53	5.58	6:5:1
2183.81	16.91	15.79	14.90	3:6:3
2199.80	1.74	3.18	4.90	4:6:2
2215.80	4.23	4.20	3.08	5:6:1
2256.82	2.08	3.04	2.17	4:7:1
2320.83	1.67	1.88	2.23	6:5:2
2361.86	3.25	2.25	3.02	5:6:2
2377.85	1.50	1.84	2.73	6:6:1
2402.88	2.05	2.20	4.26	4:7:2
2418.88	0.97	1.54	-	5:7:1
2466.89	1.03	-	-	6:5:3
2507.91	2.04	2.48	-	5:6:3
2523.91	1.58	1.73	1.47	6:6:2
2539.90	2.48	4.79	9.56	7:6:1
2548.94	1.26	1.14	0.66	4:7:3
2580.93	1.87	2.07	2.48	6:7:1
2685.96	2.74	3.39	4.30	7:6:2
2726.99	2.55	3.12	-	6:7:2
2768.01	1.35	-	-	5:8:2
2832.02	2.14	3.06	1.91	7:6:3
2873.05	1.70	1.81	1.63	6:7:3
2889.04	1.14	0.67	-	7:7:2
2978.08	0.89	0.99	2.39	7:6:4
3019.10	1.09	1.26	-	6:7:4

Table VIII.

FT activities
(nmol/hr/mg protein)

	α1,2 FT	α1,3 FT	α1,6 FT	GalT
CHO	< 0.01	0.03	4.31	12.5
PER.C6	< 0.01	0.65	3.62	3.41

CLAIMS

1. A method for identifying a mammalian cell capable of producing a proteinaceous molecule comprising a predetermined post-translational modification, said method comprising the steps of:
   a) analyzing the post-translational modification on a protein produced by said mammalian cell; and
   b) determining whether said protein comprises said predetermined post-translational modification.

2. A method for selecting a mammalian cell capable of producing a proteinaceous molecule comprising a predetermined post-translational modification, said method comprising the steps of:
   a) analyzing the presence or absence of a tissue specific marker or a combination of tissue specific markers in said mammalian cell or on the cell surface of said mammalian cell, which marker or combination of said markers is indicative for said predetermined post-translational modification to be present on said proteinaceous molecule; and
   b) selecting said mammalian cell on the basis of the presence or absence of said tissue specific markers.

3. A method for obtaining a mammalian cell from a heterogeneous cell population, said mammalian cell being capable of producing a proteinaceous molecule comprising a predetermined post-translational modification, said method comprising the steps of:

a) sorting cells on the basis of the post-translational modifications on proteins produced by said cells in said heterogeneous cell population; and

b) selecting the cells capable of producing proteins comprising said predetermined post-translational modification.

4. A method according to any one of claims 1-3, wherein said predetermined post-translational modification comprises glycosylation.

5. A method according to claim 4, wherein said glycosylation comprises at least one modification selected from the group consisting of a Lewis x, a sialyl Lewis x, a GalNac structure, a GlcNac structure, a LacdiNAc structure, a α1,3-linked fucose attached to N-acetyl-glucosamine, a terminal N-acetyl-glucosamine, a terminal galactose, a bisecting N-acetyl-glucosamine, a sulphate group and a sialic acid.

6. A method according to any one of claims 1-5, wherein said mammalian cell is of neural origin.

7. A method according to any one of claims 1-6, wherein said mammalian cell is a human cell.

8. A method according to anyone of claims 1-7, wherein said mammalian cell has been immortalized.

9. A method according to any one of claims 1-8, wherein said mammalian cell has been provided with a nucleic acid encoding the E1 region, or a part thereof, from human adenovirus in such a way that said mammalian cell harbors the nucleic acid in an expressible form.

10. A method for identifying a mammalian cell capable of producing a proteinaceous molecule comprising a predetermined post-translational modification, said method comprising the steps of:
a) providing said mammalian cell with a nucleic acid encoding a protein capable of comprising post-translational modifications, in such a way that said mammalian cell harbors said nucleic acid in an expressible form;
b) culturing said mammalian cell under conditions conducive to the production of said protein;
c) analyzing the post-translational modification on said protein produced by said mammalian cell; and
d) determining whether said post-translational modification present on said protein comprises said predetermined post-translational modification.

11. A method for identifying a mammalian cell capable of producing a proteinaceous molecule comprising a predetermined post-translational modification, said method comprising the steps of:
a) providing said mammalian cell with a nucleic acid encoding said proteinaceous molecule capable of comprising post-translational modifications, in

such a way that said mammalian cell harbors said nucleic acid in an expressible form;

b) culturing said mammalian cell under conditions conducive to the production of said proteinaceous molecule;

c) analyzing the post-translational modification on said proteinaceous molecule produced by said mammalian cell; and

d) determining whether said post-translational modification present on said proteinaceous molecule comprises said predetermined post-translational modification.

12. A method according to claim 10 or 11, wherein said protein is erythropoietin.

13. A method according to claim 10 or 11, wherein said protein is a monoclonal antibody, or a part thereof.

14. A method according to any one of claims 10-13, wherein said predetermined post-translational modification comprises glycosylation.

15. A method according to claim 14, wherein said glycosylation comprises at least one modification selected from the group consisting of a Lewis x, a sialyl Lewis x, a GalNac structure, a GlcNac structure, a LacdiNAc structure, a α1,3-linked fucose attached to N-acetyl-glucosamine, a terminal N-acetyl-glucosamine, a terminal galactose, a bisecting N-acetyl-glucosamine, a sulphate group and a sialic acid.

16. A method according to any one of claims 10-15, wherein said mammalian cell is of neural origin.

17. A method according to any one of claims 10-16, wherein said mammalian cell is a human cell.

18. A method according to anyone of claims 10-17, wherein said mammalian cell has been immortalized.

19. A method according to any one of claims 10-16, wherein said mammalian cell has been provided with a nucleic acid encoding the E1 region, or a part thereof, from human adenovirus in such a way that said mammalian cell harbors the nucleic acid in an expressible form.

20. A method for producing a proteinaceous molecule comprising a predetermined post-translational modification, comprising the steps of:

a) providing a mammalian cell obtainable by a method according to any one of claims 1-9, with a nucleic acid encoding said proteinaceous molecule in such a way that said mammalian cell harbors said nucleic acid in an expressible form; and

b) culturing said mammalian cell under conditions conducive to the production of said proteinaceous molecule,

wherein said proteinaceous molecule is not human erythropoietin or a fully human monoclonal antibody.

21. A method according to claim 20, wherein said brain-type modification comprises glycosylation.

22. A method according to claim 21, wherein said glycosylation comprises at least one modification selected from the group consisting of a Lewis x, a sialyl Lewis x, a GalNac structure, a GlcNac structure, a LacdiNAc structure, a α1,3-linked fucose attached to N-acetyl-glucosamine, a terminal N-acetyl-glucosamine, a terminal galactose, a bisecting N-acetyl-glucosamine, a sulphate group and a sialic acid.

23. A method for producing a proteinaceous molecule comprising a predetermined post-translational modification, comprising the steps of:

a) identifying a mammalian cell having the ability to provide said proteinaceous molecule with said predetermined post-translational modification;

b) providing said mammalian cell with a nucleic acid encoding said proteinaceous molecule in such a way that said mammalian cell harbors said nucleic acid in an expressible form; and

c) culturing said mammalian cell under conditions conducive to the production of said proteinaceous molecule.

24. A method for producing a proteinaceous molecule comprising a predetermined post-translational modification, said method comprising the steps of:

a) identifying a mammalian cell having the ability to provide said proteinaceous molecule with said predetermined post-translational modification;
b) providing said mammalian cell with a nucleic acid encoding said proteinaceous molecule in such a way that said mammalian cell harbors said nucleic acid in an expressible form;
c) culturing said mammalian cell under conditions conducive to the production of said proteinaceous molecule;
d) analyzing said post-translational modifications on said proteinaceous molecule so produced; and
e) determining whether said post-translational modification present on said proteinaceous molecule comprises said predetermined post-translational modification.

25. A method according to claim 23 or 24, comprising the extra step of purifying said proteinaceous molecule from the mammalian cell culture.

26. A method according to any of claims 23-25, wherein said proteinaceous molecule is erythropoietin.

27. A method according to any of claims 23-25, wherein said proteinaceous molecule is a monoclonal antibody, or a part thereof.

28. A method according to any one of claims 23-27, wherein said predetermined post-translational modification comprises glycosylation.

29. A method according to claim 28, wherein said glycosylation comprises at least one modification selected from the group consisting of a Lewis x, a sialyl Lewis x, a GalNac structure, a GlcNac structure, a LacdiNAc structure, a α1,3-linked fucose attached to N-acetyl-glucosamine, a terminal N-acetyl-glucosamine, a terminal galactose, a bisecting N-acetyl-glucosamine, a sulphate group and a sialic acid.

30. A method according to any one of claims 23-29, wherein said mammalian cell is of neural origin.

31. A method according to any one of claims 23-30, wherein said mammalian cell is a human cell.

32. A method according to anyone of claims 23-31, wherein said mammalian cell has been immortalized prior or subsequent to step a).

33. A method according to any one of claims 23-32, wherein said mammalian cell has been provided with a nucleic acid encoding the E1 region, or a part thereof, from human adenovirus in such a way that said mammalian cell harbors the nucleic acid in an expressible form.

34. A method according to claim 33, wherein said mammalian cell is PER.C6, deposited under No. 96022940 at the European Collection of Animal Cell Cultures at the Center for Applied Microbiology and Research.

35. A method for producing a proteinaceous molecule comprising a predetermined post-translational modification, said method comprising the steps of:
a) providing a mammalian cell obtainable by a method according to any one of claims 1-9, with a nucleic acid encoding said proteinaceous molecule in such a way that said mammalian cell harbors said nucleic acid in an expressible form;
b) culturing said mammalian cell under conditions conducive to the production of said proteinaceous molecule, and
c) purifying said proteinaceous molecule from the mammalian cell culture.

36. A method for producing a proteinaceous molecule comprising a predetermined post-translational modification, said method comprising the steps of:
a) providing a mammalian cell obtainable by a method according to any one of claims 1-9, with a nucleic acid encoding said proteinaceous molecule in such a way that said mammalian cell harbors said nucleic acid in an expressible form;
b) culturing said mammalian cell under conditions conducive to the production of said proteinaceous molecule;
c) analyzing said post-translational modifications on said proteinaceous molecule so produced; and
d) determining whether said post-translational modification present on said proteinaceous molecule comprises said predetermined post-translational modification.

37. A method according to claim 36, comprising the extra step of purifying said proteinaceous molecule from the mammalian cell culture.

38. A method according to any one of claims 35-37, wherein said proteinaceous molecule is erythropoietin.

39. A method according to any one of claims 35-37, wherein said proteinaceous molecule is a monoclonal antibody, or a part thereof.

40. A method according to any one of claims 35-39, wherein said predetermined post-translational modification comprises glycosylation.

41. A method according to claim 40, wherein said glycosylation comprises at least one modification selected from the group consisting of a Lewis x, a sialyl Lewis x, a GalNac structure, a GlcNac structure, a LacdiNAc structure, a α1,3-linked fucose attached to N-acetyl-glucosamine, a terminal N-acetyl-glucosamine, a terminal galactose, a bisecting N-acetyl-glucosamine, a sulphate group and a sialic acid.

42. A method according to claim 25, wherein said purification comprises a step that employs said predetermined post-translational modification.

43. A method according to claim 42, wherein said proteinaceous molecule is erythropoietin.

44. A method according to claim 42, wherein said proteinaceous molecule is monoclonal antibody, or a part thereof.

45. A method according to any one of claims 42-44, wherein said predetermined post-translational modification comprises glycosylation.

46. A method according to claim 45, wherein said glycosylation comprises at least one modification selected from the group consisting of a Lewis x, a sialyl Lewis x, a GalNac structure, a GlcNac structure, a LacdiNAc structure, a α1,3-linked fucose attached to N-acetyl-glucosamine, a terminal N-acetyl-glucosamine, a terminal galactose, a bisecting N-acetyl-glucosamine, a sulphate group and a sialic acid.

47. A method according to any one of claims 42-46, wherein said mammalian cell is of neural origin.

48. A method according to any one of claims 42-47, wherein said mammalian cell is a human cell.

49. A method according to any one of claims 42-48, wherein said mammalian cell has been immortalized prior or subsequent to step a).

50. A method according to any one of claims 42-49, wherein said mammalian cell has been provided with a nucleic acid encoding the E1 region, or a part thereof, from human adenovirus in such a way that said mammalian

cell harbors the nucleic acid in an expressible form.

51. A method according to claim 50, wherein said mammalian cell is PER.C6, deposited under No. 96022940 at the European Collection of Animal Cell Cultures at the Center for Applied Microbiology and Research.

52. A method according to claim 35 or 37, wherein said purification comprises a step that employs said predetermined post-translational modification.

53. A method according to claim 52, wherein said predetermined post-translational modification comprises glycosylation.

54. A method according to claim 53, wherein said glycosylation comprises at least one modification selected from the group consisting of a Lewis x, a sialyl Lewis x, a GalNac structure, a GlcNac structure, a LacdiNAc structure, a α1,3-linked fucose attached to N-acetyl-glucosamine, a terminal N-acetyl-glucosamine, a terminal galactose, a bisecting N-acetyl-glucosamine, a sulphate group and a sialic acid.

55. A method according to any one of claims 42-54, wherein said purification comprises a step in which an antibody is employed that is specific for an epitope present in said predetermined post-translational modification.

56. A method according to any one of claims 42-55, wherein said proteinaceous molecule is purified to homogeneity.

57. A pharmaceutically acceptable composition comprising a proteinaceous molecule having a predetermined post-translational modification, obtainable according to any one of claims 20-56, and a pharmaceutically acceptable carrier.

58. A pharmaceutically acceptable composition according to claim 57, wherein said proteinaceous molecule is erythropoietin.

59. A pharmaceutically acceptable composition according to claim 58, wherein said erythropoietin has a lower erythropoietic effect as compared to erythropoietin not having said predetermined post-translational modification.

60. Recombinantly produced erythropoietin comprising at least one post-translational modification selected from the group consisting of: a sialyl Lewis x structure, a Lewis x structure, a α1,3-linked fucose attached to N-acetyl-glucosamine, a LacdiNAc structure, a terminal N-acetyl-glucosamine group and a terminal galactose group.

61. Recombinantly produced erythropoietin according to claim 60, wherein said erythropoietin is produced on a mammalian cell obtainable according to any one of claims 1-11.

62. Use of PER.C6 for the production of a proteinaceous molecule comprising a predetermined post-translational modification.

63. Use of PER.C6 according to claim 62, wherein said proteinaceous molecule is erythropoietin.

64. Use of PER.C6 according to claim 62, wherein said proteinaceous molecule is a monoclonal antibody, or a part thereof.

65. A proteinaceous molecule obtainable according to any one of claims 20-56 for the treatment of a human or a human body by surgery, therapy or diagnosis.

66. Use of a proteinaceous molecule obtainable according to any one of claims 20-56 for the manufacture of a medicament for the treatment of hypoxia-induced disorders, neurodegenerative afflictions, or acute damage to the central- or peripheral nervous system.

67. Use of a proteinaceous molecule obtainable according to anyone of claims 20-56 for the manufacture of a medicament for the treatment of ischemia/reperfusion injuries.

68. Use of recombinantly produced erythropoietin according to claim 60 or 61 for the preparation of a medicament for the treatment of hypoxia-induced disorders, neurodegenerative afflictions, or acute damage to the central- or peripheral nervous system.

69. Use of recombinantly produced erythropoietin according to claim 60 or 61 for the preparation of a medicament for the treatment of ischemia/reperfusion injuries.

70. Use of recombinantly produced erythropoietin according to claim 60 or 61 for the preparation of a medicament for the treatment of inflammatory disease.

71. Use of recombinantly produced erythropoietin according to claim 60 or 61 for the preparation of a medicament for the treatment of an immune disorder.

72. Use of a composition of erythropoietin-like molecules selected from the group consisting of one or more muteins of erythropoietin, one or more derivatives of erythropoietin, and a composition of one or more fractions of erythropoietin molecules sialylated to a varying degree, for the preparation of a medicament for the treatment of a disorder selected from the group consisting of ischemia, a reperfusion injury, a hypoxia-induced disorder, an inflammatory disease, a neurodegenerative disorder, and acute damage to the central- or peripheral nervous system, wherein said composition of erythropoietin-like molecules has on a protein content basis a lower erythropoietic activity *in vivo* than epoetin alfa.

73. Use of a composition of erythropoietin-like molecules selected from the group consisting of one or more

muteins of erythropoietin, one or more derivatives of erythropoietin, and a composition of one or more fractions of erythropoietin molecules sialylated to a varying degree, for the preparation of a medicament for the treatment of a disorder selected from the group consisting of ischemia, a reperfusion injury, a hypoxia-induced disorder, an inflammatory disease, a neurodegenerative disorder or acute damage to the central or peripheral nervous system, wherein said composition of erythropoietin-like molecules is characterized by an average number of sialic acid residues that is at least 10% lower than the average number of sialic acid residues per erythropoietin molecule in epoetin alfa.

74. Use according to claim 73, wherein the average number of sialic acid residues per erythropoietin-like molecule is at least 20% lower than the average number of sialic acid residues per molecule in epoetin alfa.

75. Use according to claim 73, wherein the average number of sialic acid residues per erythropoietin-like molecule is at least 30% lower than the average number of sialic acid residues per molecule in epoetin alfa.

76. Use according to claim 73, wherein the average number of sialic acid residues per erythropoietin-like molecule is at least 40% lower than the average number of sialic acid residues per molecule in

epoetin alfa.

77. Use according to claim 73, wherein the average number of sialic acid residues per erythropoietin-like molecule is at least 50% lower than the average number of sialic acid residues per molecule in epoetin alfa.

78. Use according to claim 73, wherein the average number of sialic acid residues per erythropoietin-like molecule is at least 60% lower than the average number of sialic acid residues per molecule in epoetin alfa.

79. Use according to claim 73, wherein the average number of sialic acid residues per erythropoietin-like molecule is at least 70% lower than the average number of sialic acid residues per molecule in epoetin alfa.

80. Use according to claim 73, wherein the average number of sialic acid residues per erythropoietin-like molecule is at least 80% lower than the average number of sialic acid residues per molecule in epoetin alfa.

81. Use according to claim 73, wherein the average number of sialic acid residues per erythropoietin-like molecule is at least 90% lower than the average number of sialic acid residues per molecule in epoetin alfa.

82. Use according to any one of claims 73-81, wherein said composition of erythropoietin-like molecules has been obtained by subjecting erythropoietin or an erythropoietin-like molecule to treatment to lower the average number of sialic acid residues per erythropoietin-like molecule.

83. Use according to claim 82, wherein said treatment comprises the step of subjecting the erythropoietin or erythropoietin-like molecule to the action of an enzyme or chemical capable of splitting a glycosidic bond.

84. Use according to any one of claims 73-81, wherein said composition of erythropoietin-like molecules has been obtained by separation from fractions comprising erythropoietin-like molecules that have a higher sialic acid content.

85. Use according to claim 72 to 83, wherein said composition of erythropoietin-like molecules is obtainable by recombinant expression of erythropoietin or a mutein of erythropoietin in transfected Chinese Hamster Ovary cells, Baby Hamster Kidney cells, or transfected human cells followed by one or more purification steps.

86. Use according to any one of claims 73-81, wherein said erythropoietin-like molecules have been produced by a method according to any one of claims 23-26.

87. Use according to any one of claims 73-81, wherein said erythropoietin-like molecules have been produced on an immortalized human cell.

88. Use of a composition of erythropoietin-like molecules selected from the group consisting of one or more muteins of erythropoietin, one or more derivatives of erythropoietin, and a composition of one or more fractions of erythropoietin molecules sialylated to a varying degree, for the preparation of a medicament for the treatment of a disorder selected from the group consisting of ischemia, a reperfusion injury, a hypoxia-induced disorder, an inflammatory disease, a neurodegenerative disorder or acute damage to the central or peripheral nervous system, wherein said composition is characterized by the presence of erythropoietin-like molecules that once administered parenterally to a human or an animal subject are cleared from the bloodstream at a faster rate than epoetin alfa.

89. A pharmaceutical preparation comprising erythropoietin-like molecules selected from the group consisting of one or more muteins of erythropoietin, one or more derivatives of erythropoietin, and a composition of one or more fractions of erythropoietin or erythropoietin-like molecules sialylated to a varying degree, said preparation being characterized in having a lower erythropoietic activity in vivo on a protein content basis than epoetin alfa.

90. A pharmaceutical preparation comprising erythropoietin-like molecules selected from the group consisting of one or more muteins of erythropoietin, one or more derivatives of erythropoietin, and a composition of one or more fractions of erythropoietin or erythropoietin-like molecules sialylated to a varying degree, wherein said erythropoietin-like molecules are characterized by an average number of sialic acid residues that is at least 10% lower than the average number of sialic acid residues per erythropoietin molecule in epoetin alfa.

91. A pharmaceutical preparation according to claim 90, wherein said erythropoietin-like molecules are characterized by an average number of sialic acid residues that is at least 20% lower than the average number of sialic acid residues per erythropoietin molecule in epoetin alfa.

92. A pharmaceutical preparation according to claim 90, wherein said erythropoietin-like molecules are characterized by an average number of sialic acid residues that is at least 30% lower than the average number of sialic acid residues per erythropoietin molecule in epoetin alfa.

93. A pharmaceutical preparation according to claim 90, wherein said erythropoietin-like molecules are characterized by an average number of sialic acid residues that is at least 40% lower than the average number of sialic acid residues per erythropoietin

molecule in epoetin alfa.

94. A pharmaceutical preparation according to claim 90, wherein said erythropoietin-like molecules are characterized by an average number of sialic acid residues that is at least 50% lower than the average number of sialic acid residues per erythropoietin molecule in epoetin alfa.

95. A pharmaceutical preparation according to claim 90, wherein said erythropoietin-like molecules are characterized by an average number of sialic acid residues that is at least 60% lower than the average number of sialic acid residues per erythropoietin molecule in epoetin alfa.

96. A pharmaceutical preparation according to claim 90, wherein said erythropoietin-like molecules are characterized by an average number of sialic acid residues that is at least 70% lower than the average number of sialic acid residues per erythropoietin molecule in epoetin alfa.

97. A pharmaceutical preparation according to claim 90, wherein said erythropoietin-like molecules are characterized by an average number of sialic acid residues that is at least 80% lower than the average number of sialic acid residues per erythropoietin molecule in epoetin alfa.

98. A pharmaceutical preparation according to claim 90, wherein said erythropoietin-like molecules are

characterized by an average number of sialic acid residues that is at least 90% lower than the average number of sialic acid residues per erythropoietin molecule in epoetin alfa.

99. A pharmaceutical preparation according to any one of claims 90-98, wherein said erythropoietin-like molecules have been obtained by subjecting erythropoietin-like molecules to treatment to lower the average number of sialic acid residues per erythropoietin-like molecule.

100. A pharmaceutical preparation according to claim 99, wherein said treatment comprises the step of subjecting the erythropoietin-like molecules to an enzyme or a chemical capable of splitting a glycosidic bond.

101. A pharmaceutical preparation according to any one of claims 90-98, wherein said erythropoietin-like molecules have been obtained by separation from fractions comprising erythropoietin-like molecules that have a higher sialic acid content.

102. A pharmaceutical preparation according to any one of claims 90-101, wherein said erythropoietin-like molecules are obtainable by recombinant expression of erythropoietin or a mutein of erythropoietin in transfected Chinese Hamster Ovary cells, Baby Hamster Kidney cells, or transfected human cells followed by one or more purification steps.

103. A pharmaceutical preparation according to any one of claims 90-98, wherein said erythropoietin-like molecules have been produced by a method according to any one of claims 23-26.

104. A pharmaceutical preparation comprising erythropoietin-like molecules selected from the group consisting of one or more muteins of erythropoietin, one or more derivatives of erythropoietin, and a composition of one or more fractions of erythropoietin or erythropoietin-like molecules sialylated to a varying degree, said preparation being characterized by the presence of erythropoietin-like molecules that once administered parenterally to a human or an animal subject are cleared from the bloodstream at a faster rate than epoetin alfa.

105. A method for the preventative and/or therapeutic treatment of a disorder selected from the group consisting of ischemia, a reperfusion injury, a hypoxia-induced disorder, an inflammatory disease, a neurodegenerative disorder, and acute damage to the central- or peripheral nervous system, said method comprising the step of administering to a human or animal subject a composition of erythropoietin-like molecules selected from the group consisting of one or more muteins of erythropoietin, one or more derivatives of erythropoietin, and a composition of one or more fractions of erythropoietin or erythropoietin-like molecules sialylated to a varying degree, wherein said composition is

characterized by the presence of erythropoietin-like molecules that once administered parenterally to a human or an animal subject are cleared from the bloodstream at a faster rate than epoetin alfa.

106. A method for the preventative and/or therapeutic treatment of a disorder selected from the group consisting of ischemia, a reperfusion injury, a hypoxia-induced disorder, an inflammatory disease, a neurodegenerative disorder, and acute damage to the central- or peripheral nervous system, said method comprising the step of administering to a human or animal subject a composition of erythropoietin-like molecules selected from the group consisting of one or more muteins of erythropoietin, one or more derivatives of erythropoietin, and a composition of one or more fractions of erythropoietin or erythropoietin-like molecules sialylated to a varying degree, wherein said composition of erythropoietin-like molecules is characterized by an average number of sialic acid residues that is at least 10% lower than the average number of sialic acid residues per erythropoietin molecule in epoetin alfa.

107. A method according to claim 106, wherein the average number of sialic acid residues per erythropoietin-like molecule is at least 20% lower than the average number of sialic acid residues per molecule in epoetin alfa.

108. A method according to claim 106, wherein the average number of sialic acid residues per erythropoietin-like molecule is at least 30% lower than the average number of sialic acid residues per molecule in epoetin alfa.

109. A method according to claim 106, wherein the average number of sialic acid residues per erythropoietin-like molecule is at least 40% lower than the average number of sialic acid residues per molecule in epoetin alfa.

110. A method according to claim 106, wherein the average number of sialic acid residues per erythropoietin-like molecule is at least 50% lower than the average number of sialic acid residues per molecule in epoetin alfa.

111. A method according to claim 106, wherein the average number of sialic acid residues per erythropoietin-like molecule is at least 60% lower than the average number of sialic acid residues per molecule in epoetin alfa.

112. A method according to claim 106, wherein the average number of sialic acid residues per erythropoietin-like molecule is at least 70% lower than the average number of sialic acid residues per molecule in epoetin alfa.

113. A method according to claim 106, wherein the average number of sialic acid residues per erythropoietin-

like molecule is at least 80% lower than the average number of sialic acid residues per molecule in epoetin alfa.

114. A method according to claim 106, wherein the average number of sialic acid residues per erythropoietin-like molecule is at least 90% lower than the average number of sialic acid residues per molecule in epoetin alfa.

115. A method according to any one of claims 106-114, wherein said erythropoietin-like molecules have been obtained by subjecting erythropoietin-like molecules to treatment to lower the average number of sialic acid residues per erythropoietin-like molecule.

116. A method according to claim 115, wherein said treatment comprises the step of subjecting erythropoietin-like molecules to the action of an enzyme or a chemical capable of splitting a glycosidic bond.

117. A method according to any one of claims 106-116, wherein said erythropoietin-like molecules have been obtained by separation from fractions comprising erythropoietin-like molecules that have a higher sialic acid content.

118. A method according to any one of claims 105-117, wherein said erythropoietin-like molecules are obtainable by recombinant expression of erythropoietin or a mutein of erythropoietin in

transfected Chinese Hamster Ovary cells, Baby Hamster Kidney cells, or transfected human cells followed by one or more purification steps.

119. A method according to any one of claims 106-114, wherein said erythropoietin-like molecules have been produced by a method according to any one of claims 23-26.

120. A method for treatment or preventing a disorder selected from the group consisting of ischemia, a reperfusion injury, a hypoxia-induced disorder, an inflammatory disease, a neurodegenerative disorder, and acute damage to the central- or peripheral nervous system, said method comprising the step of administering to a human or animal subject a composition of erythropoietin-like molecules selected from the group consisting of one or more muteins of erythropoietin, one or more derivatives of erythropoietin, or a composition of one or more fractions of erythropoietin molecules sialylated to a varying degree, wherein said composition is characterized by the presence of erythropoietin-like molecules that once administered parenterally to a human or an animal subject are cleared from the bloodstream at a faster rate than epoetin alfa.

121. A composition of erythropoietin-like molecules selected from the group consisting of one or more muteins of erythropoietin, one or more derivatives of erythropoietin, and a composition of one or more fractions of erythropoietin or erythropoietin-like

molecules sialylated to a varying degree, for the preventative and/or therapeutic treatment of the human or animal body, wherein said composition of erythropoietin-like molecules has on a protein content basis a lower erythropoietic activity *in vivo* than epoetin alfa.

122. A composition of erythropoietin-like molecules selected from the group consisting of one or more muteins of erythropoietin, one or more derivatives of erythropoietin, and a composition of one or more fractions of erythropoietin or erythropoietin-like molecules sialylated to a varying degree, for the preventative and/or therapeutic treatment of the human or animal body, wherein said composition of erythropoietin-like molecules is characterized by an average number of sialic acid residues that is at least 10% lower than the average number of sialic acid residues per erythropoietin molecule in epoetin alfa.

123. A composition according to claim 122 for the preventative and/or therapeutic treatment of the human or animal body, wherein the average number of sialic acid residues per erythropoietin-like molecule is at least 20% lower than the average number of sialic acid residues per molecule in epoetin alfa.

124. A composition according to claim 122 for the preventative and/or therapeutic treatment of the human or animal body, wherein the average number of

sialic acid residues per erythropoietin-like molecule is at least 30% lower than the average number of sialic acid residues per molecule in epoetin alfa.

125. A composition according to claim 122 for the preventative and/or therapeutic treatment of the human or animal body, wherein the average number of sialic acid residues per erythropoietin-like molecule is at least 40% lower than the average number of sialic acid residues per molecule in epoetin alfa.

126. A composition according to claim 122 for the preventative and/or therapeutic treatment of the human or animal body, wherein the average number of sialic acid residues per erythropoietin-like molecule is at least 50% lower than the average number of sialic acid residues per molecule in epoetin alfa.

127. A composition according to claim 122 for the preventative and/or therapeutic treatment of the human or animal body, wherein the average number of sialic acid residues per erythropoietin-like molecule is at least 60% lower than the average number of sialic acid residues per molecule in epoetin alfa.

128. A composition according to claim 122 for the preventative and/or therapeutic treatment of the human or animal body, wherein the average number of

sialic acid residues per erythropoietin-like molecule is at least 70% lower than the average number of sialic acid residues per molecule in epoetin alfa.

129. A composition according to claim 122 for the preventative and/or therapeutic treatment of the human or animal body, wherein the average number of sialic acid residues per erythropoietin-like molecule is at least 80% lower than the average number of sialic acid residues per molecule in epoetin alfa.

130. A composition according to claim 122 for the preventative and/or therapeutic treatment of the human or animal body, wherein the average number of sialic acid residues per erythropoietin-like molecule is at least 90% lower than the average number of sialic acid residues per molecule in epoetin alfa.

131. A composition according to any one of claims 122-130 for the preventative and/or therapeutic treatment of the human or animal body, wherein said composition of erythropoietin-like molecules has been obtained by subjecting erythropoietin-like molecules to treatment with an enzyme such as neuraminidase or a chemical substance such as an acid to lower the average number of sialic acid residues per erythropoietin-like molecule.

132. A composition according to any one of claims 122-130 for the preventative and/or therapeutic treatment of the human or animal body, wherein said composition of erythropoietin-like molecules has been obtained by separation from fractions comprising erythropoietin-like molecules that have a higher sialic acid content.

133. A composition according to any one of claim 121-132 for the preventative and/or therapeutic treatment of the human or animal body, wherein said erythropoietin-like molecules are obtainable by recombinant expression of erythropoietin or a mutein of erythropoietin in transfected Chinese Hamster Ovary cells, Baby Hamster Kidney cells, or transfected human cells followed by one or more purification steps.

134. A composition according to any one of claims 122-130 for the preventative and/or therapeutic treatment of the human or animal body, wherein said erythropoietin-like molecules have been produced by a method according to any one of claims 23-26 for the preventative and/or therapeutic treatment of the human or animal body.

135. A composition of erythropoietin-like molecules selected from the group consisting of one or more muteins of erythropoietin, one or more derivatives of erythropoietin, and a composition of one or more fractions of erythropoietin or erythropoietin-like molecules sialylated to a varying degree, for the

preventative and/or therapeutic treatment of the human or animal body, wherein said composition of erythropoietin-like molecules is characterized by the presence of erythropoietin-like molecules that once administered parenterally to a human or an animal subject are cleared from the bloodstream at a faster rate than epoetin alfa.

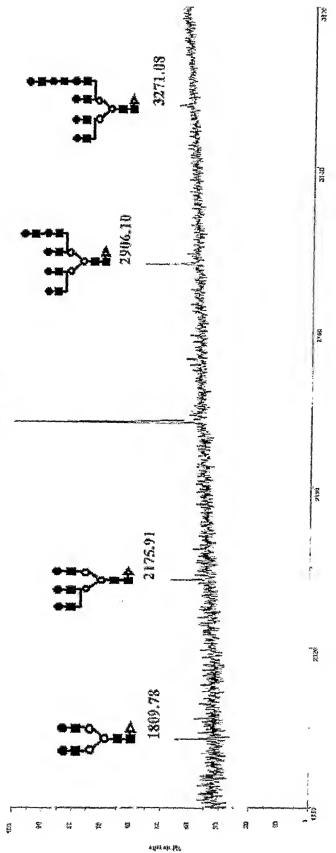
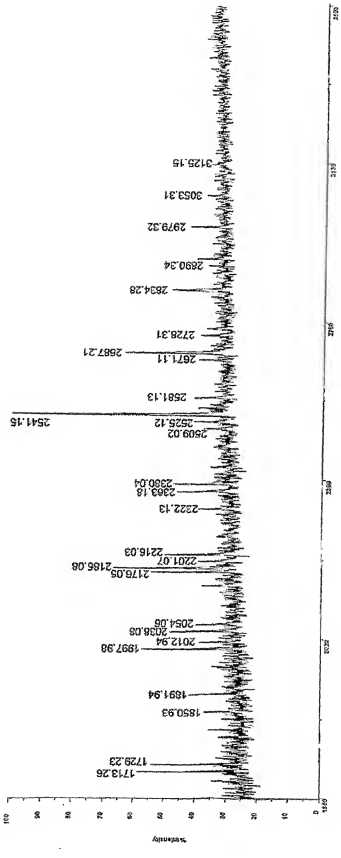


Fig. 1A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2509.02
2525.12
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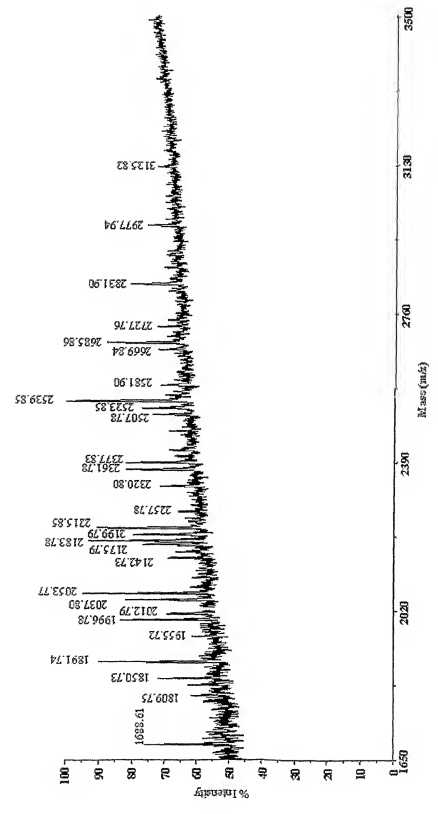
Fig. 1B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80
70
60
50
40
30
20
10
0
1659
2020
2380
2740
3100
3460
Mass (m/z)
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1809.75
1850.73
1891.74
1955.72
1996.78
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2183.78
2199.79
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2507.78
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2581.90
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2685.86
2727.76
2831.90
2977.94
3125.82

Fig. 1C

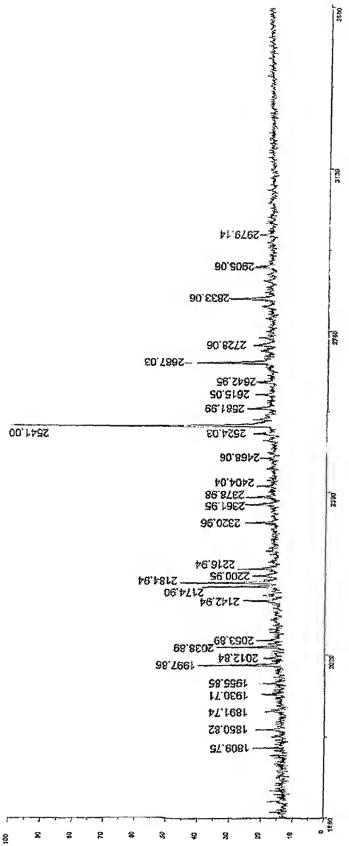

1809.75
1850.82
1891.74
1930.71
1955.85
1997.86
2013.84
2038.89
2053.89
2142.94
2174.90
2184.94
2200.95
2216.94
2320.96
2361.95
2378.98
2404.04
2468.06
2524.03
2541.00
2561.99
2615.05
2642.95
2667.03
2728.06
2833.06
2905.06
2979.14

Fig. 1D

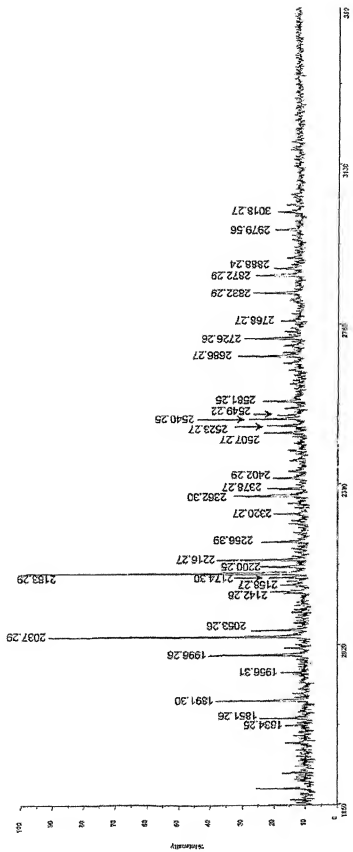

Intensity
100
90
80
70
60
50
40
30
20
10
0
1850
2050
2250
2450
2650
2850
3050
3250
3018.27
2979.56
2888.24
2872.29
2832.29
2768.27
2726.26
2686.27
2561.25
2549.22
2540.25
2523.27
2507.27
2402.29
2378.27
2362.30
2320.27
2266.39
2216.27
2200.25
2174.30
2159.27
2142.26
2153.29
2053.26
2037.29
1996.26
1956.31
1891.30
1851.26
1834.25

Fig. 1E

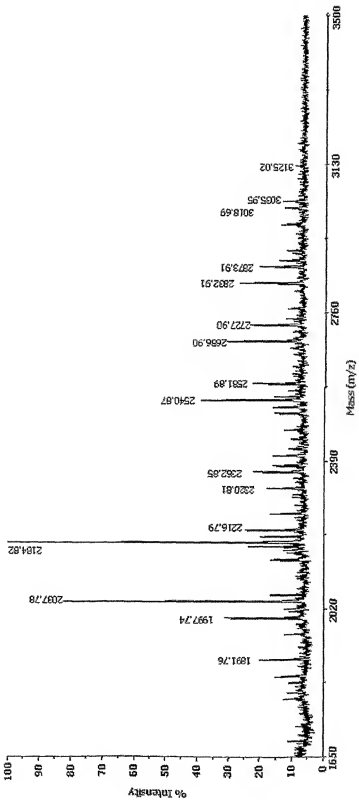

% Intensity
100
90
80
70
60
50
40
30
20
10
0
1630
2020
2390
2760
3130
3500
Mass (m/z)
1891.76
1937.74
2037.78
2184.82
2216.79
2320.81
2362.85
2540.67
2581.89
2686.90
2727.90
2882.91
2873.91
3018.69
3035.95
3125.02

Fig. 1F

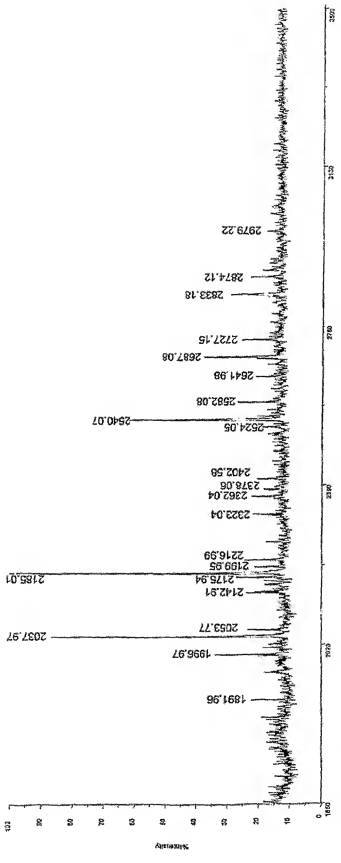

1891.96
1996.97
2037.97
2053.77
2142.91
2175.94
2185.01
2199.95
2216.59
2323.04
2362.04
2378.06
2402.56
2524.05
2540.07
2582.08
2641.98
2687.08
2727.15
2833.18
2874.12
2979.22
%Intensity
100
90
80
70
60
50
40
30
20
10
0
1850
2100
2350
2600
2850
3100
3350

Fig. 1G

Fig. 2

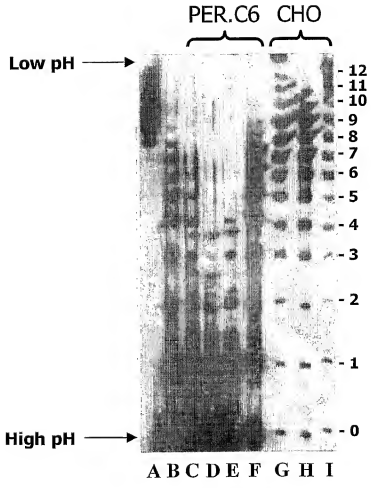


Fig. 3

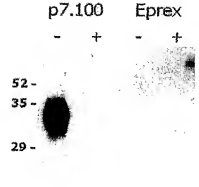


Fig. 4

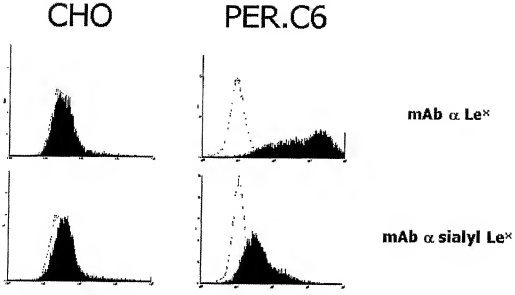
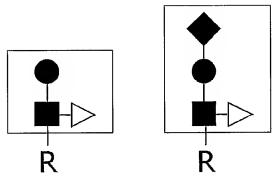
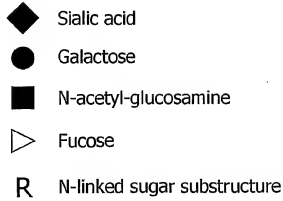


Fig. 5


R
R
Lewis x
Sialyl-Lewis x


Sialic acid
Galactose
N-acetyl-glucosamine
Fucose
R
N-linked sugar substructure

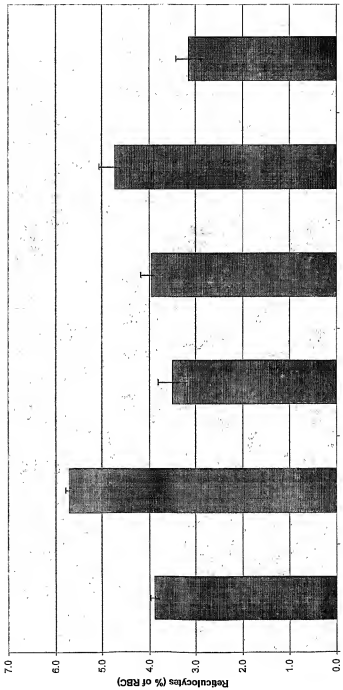

Biological activity of PER.C6-EPO
Reticulocytes (% of RBC)
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0.0
Eprex 1 eU
Eprex 6 eU
P7 EPO 6 eU
P7 EPO 25 eU
P7 EPO 125 eU
PBS/0.05% Tween
Treatment

Fig. 6

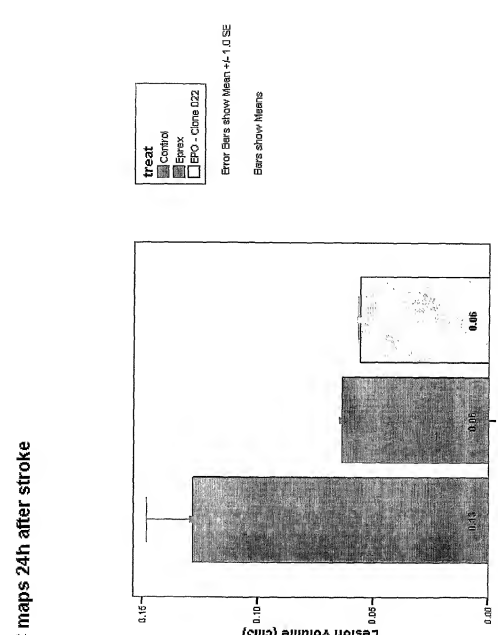

ADC maps 24h after stroke
Lesion volume (cm3)
0.15
0.10
0.05
0.00
0.13
0.10
0.06
day
treat
Control
Eprex
EPO - clone 022
Error Bars show Mean +/- 1.0 SE
Bars show Means

Fig. 7A

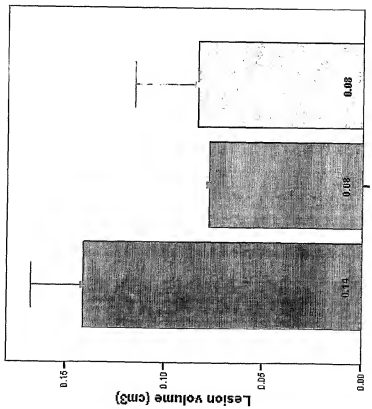

T2 maps 24h after stroke
treat
Control
Eprex
EPO - Clone 022
Error Bars show Mean +/- 1.0 SE
Bars show Means
Lesion volume (cm3)
0.15
0.10
0.05
0.00
0.13
0.118
0.08
1
day

Fig. 7B

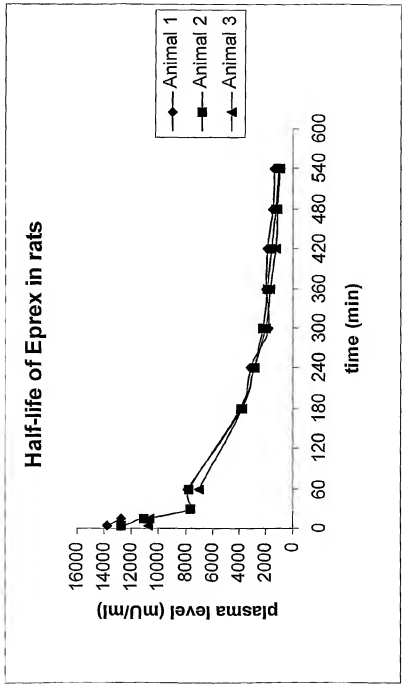

Half-life of Eprex in rats
plasma level (mU/ml)
16000
14000
12000
10000
8000
6000
4000
2000
0
0 60 120 180 240 300 360 420 480 540 600
time (min)
Animal 1
Animal 2
Animal 3

Fig. 8

INTERNATIONAL SEARCH REPORT

International Application No
PCT/NL 02/00257

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C07K14/505 A61K38/18

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, BIOSIS, PAJ, WPI Data, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 00 63403 A (SCHOUTEN GOVERT JOHAN ;HATEBOER GUUS (NL); BOUT ABRAHAM (NL); INTR) 26 October 2000 (2000-10-26)	1-65
Y	the whole document	66-112, 119-135
X	US 5 856 298 A (STRICKLAND THOMAS WAYNE) 5 January 1999 (1999-01-05)	1-33, 35-50, 52-61
Y	the whole document	66-112, 119-135
Y	WO 00 61164 A (KENNETH S WARREN LAB) 19 October 2000 (2000-10-19) the whole document	66-112, 119-135

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents :

A document defining the general state of the art which is not considered to be of particular relevance

E earlier document but published on or after the international filing date

L document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

O document referring to an oral disclosure, use, exhibition or other means

P document published prior to the international filing date but later than the priority date claimed

T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

& document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
21 November 2002	03/12/2002

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Wimmer, G

Form PCT/ISA/210 (second sheet) (July 1992)

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

Continuation of Box I.1

Although claims 105-120 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the composition.

Continuation of Box I.1

Rule 39.1(iv) PCT - Method for treatment of the human or animal body by therapy

INTERNATIONAL SEARCH REPORT

International application No.
PCT/NL 02/00257

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: –
because they relate to subject matter not required to be searched by this Authority, namely:

see FURTHER INFORMATION sheet PCT/ISA/210

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

[redacted]national Application No
PCT/NL 02/00257

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
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